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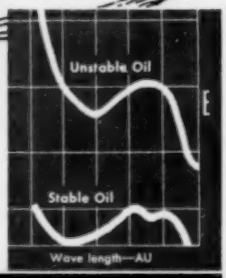

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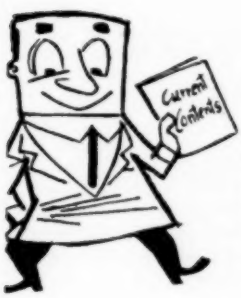

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Cinnoline Chemistry IV*

Infrared Spectra

By RAYMOND N. CASTLE, DAVID B. COX†, and JOHN F. SUTTLE‡

The infrared spectra of eighteen cinnolines have been determined. A consistent absorption band at approximately 6.35 microns has been observed and this has been assigned to the interaction effects of the ring double bonds of the cinnoline ring, namely the —C=C—, the —N=N— and the —C=N— bonds. This absorption band appears to be characteristic of the cinnoline ring system.

THE SYNTHESIS of compounds containing the cinnoline ring system has been a subject of interest in this laboratory for some time (1, 2, 3). The compounds described in this paper were prepared for pharmacological screening. In the course of the preparation of the compounds described (3) it became desirable to study the infrared spectra of a number of cinnolines.

Attempts to hydrolyze α-4-cinnolyldiphenylacetonitrile to the corresponding carboxylic acid which was then expected to decarboxylate to α-4-cinnolyldiphenylmethane gave back a quantitative yield of the starting material. Analytical data indicated the presence of the nitrile group. This was confirmed by a weak but definite absorption band at 4.5 microns indicative of the —C≡N stretching vibration (Table I). These data confirm the presence of the nitrile group. Presumably this particular nitrile group is too hindered for hydrolysis to take place, even when heated sixty hours in 60 per cent sulfuric acid solution.

The infrared spectra of 18 cinnolines have been determined. In all the cinnolines studied an absorption band occurs in the 6.35 micron region. Examination of the data in Table I under the heading Cinnoline Ring will disclose absorptions varying from 6.20 microns to 6.40 microns with the majority falling between 6.34 and 6.38 microns. It is expected that interactions of the other groups present in the compounds would shift the absorption bands somewhat. This absorption band has been assigned, on the basis of the 18 compounds studied, to the interaction effects of the ring double bonds. The overall absorption at this wavelength is probably due to the combined interaction effects of —N=N—, —N=C— and —C=C— bonds. The absorption spectrum of liquid pyridazine (4) shows a very strong absorption band at 6.38 microns (1567 $cm.^{-1}$).

The spectrum of 4-hydroxycinnoline fails to reveal on OH stretching vibration in the 3 micron region, however, a shoulder is found at 6.17 microns which is interpreted to indicate a ring, conjugated carbonyl. These data suggest that 4-hydroxycinnoline exists in the tautomeric 4-cinnolone structure, II, in the solid state. This is in agreement with the work of Hearn, Morton, and Simpson (5) in which 4-hydroxycinnoline was described as approximately a 70–30 tautomeric mixture of forms I and II with the cinnolone structure predominating in alcoholic

* Received July 28, 1958, from the University of New Mexico Laboratory of Pharmaceutical Chemistry and the Department of Chemistry, Albuquerque.

Presented before the Section of Pharmaceutical Chemistry and Biochemistry, The Fourth Pan-American Congress of Pharmacy and Biochemistry, November 4, 1957, Washington, D. C.

The authors wish to express appreciation to the Upjohn Company, Kalamazoo, Mich., for generous financial support during the course of this work.

† Upjohn Research Fellow in Chemistry. Present address: Socony Mobile Oil Laboratories, 412 Greenpoint Avenue, Brooklyn 22, N. Y.

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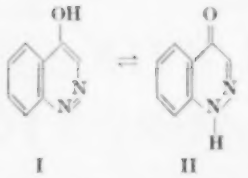
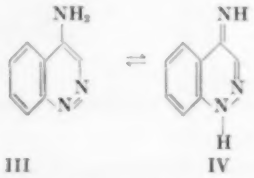


I II

solution. The spectral evidence for the structure of 6,7-dimethoxy-4-hydroxycinnoline is in agreement with the 4-cinnolone structure. There is no apparent OH stretching vibration.

The spectrum of 4-aminocinnoline reveals weak absorption band at 3.02 microns. In contrast a very strong absorption band is noted at 5.97 microns which is indicative of the —C=NH grouping. Three other absorption bands at wavelengths that can be attributed to NH bending vibrations are noted at 8.97 (m), 12.23 (m), and 13.05 (m). A fourth at 12.94 (s) can be attributed to N—H deformation vibration. Based on this evidence, 4-aminocinnoline, III should exist predominantly in the imino structure, IV, in the solid state. This interpretation is in agreement with the conclusions reached by Hearn, Morton, and Simpson (5) based upon ultraviolet absorption spectra.



III IV

The absorption band due to the nitrile group in α-4-cinnolyldiphenylacetonitrile is shifted from 4.65 microns to 4.50 microns in this strongly hindered nitrile. The intensity of this absorption is weaker than any other nitrile studied. Four strong intensity (13.17, 13.38, 14.21, and 14.32)

TABLE I.—INFRARED SPECTRA OF CINNOLINES

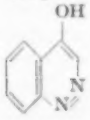
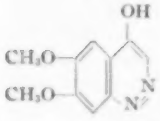
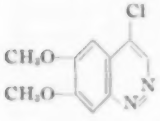
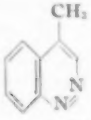
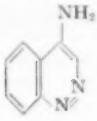
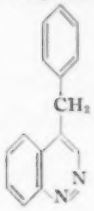
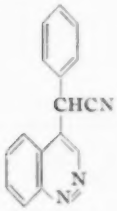
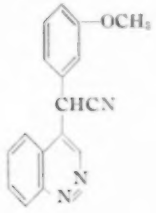
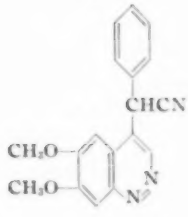
Compound	Wave Length in Microns: Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group	Miscellaneous
		6.40 sh			6.17 sh alpha, beta-unsaturated aminoketone 9.27 m
		6.30 ? sh 6.33 ? w	7.80 s 7.88 s	6.17 m 9.24 m 8.13 s 8.23 s 8.52 m 8.93 m	11.55 m 12.02 m 12.20 m 13.75
		6.20 ? w		7.72 s 7.95 m 8.04 m 8.20 m 8.34 m 8.63 m 8.81 m	6.53 6.69 9.77 w 9.95 w 10.43 m 11.76 m 12.10 m 12.57 m C—Cl
		6.37 w			6.67 w methyl deformation 8.68 w methyl rocking vibration 12.22 w methyl rocking vibration 12.82 w 13.22 14.25 w
		6.38 s			3.02 w NH stretching 5.97 s C=NH 6.63 s 7.70 s 7.93 s 8.23 s 8.97 m NH—bending 9.26 m 9.85 m 11.65 m 12.23 m NH bending 12.94 m NH deformation 13.05 s NH bending

TABLE I (*Continued*)

Compound	Wave Length in Microns					
	Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group	Miscellaneous	
		6.35 m	6.69 m		12.95 m 13.21 s 13.97 s 14.30 m	out of plane C—H deformation vibration
	4.64 s	6.34 s	6.20 w 6.59 s		3.12 s 8.12 m 8.80 m 9.18 m 13.00 m 13.15 m 13.40 m 14.25 s	out of plane C—H deformation vibration
	4.64 m	6.35 m	6.25 m 6.68 m	7.80 s 7.95 s 8.06 m 8.52 m 8.67 w 8.95 w	9.85 m 12.03 m 12.40 w 13.00 m	out of plane C—H deformation vibration
	4.65 s	6.37 s	6.20 w 6.30 m 6.63 s	7.85 m 8.04 m 8.29 m 8.81 m	12.70 w 12.95 m 13.45 w 14.25 m	out of plane C—H deformation vibration
	4.68 w	6.40 w	6.19 m	7.72 s 7.95 m 8.05 s 8.19 s 8.34 s 8.63 s 8.82 s	9.77 w 9.95 m 10.42 m 11.75 m 12.10 m 12.57 m	out of plane C—H deformation vibration

and two medium intensity (14.05 and 14.70) absorption bands are found in the spectrum of this hindered nitrile. These are attributed to the out of plane C—H deformation vibrations to be expected in a molecule of this type.

EXPERIMENTAL

The infrared spectra were determined from pastes in Nujol with a Perkin Elmer Model 21 double beam spectrophotometer.

The compounds were prepared by methods previously described (1, 2, 3) except for 4-aminocinnoline.

4-Aminocinnoline.—Three grams of 4-chlorocinnoline was heated for eight hours at 95° in a capped "coke" bottle with 150 ml. of 28% aqueous ammonia. The reaction mixture was evaporated under reduced

TABLE I (*Continued*)

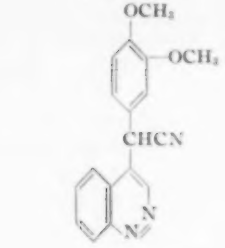
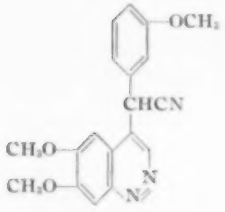
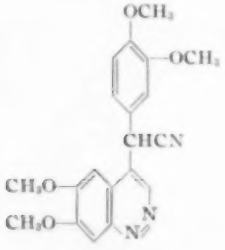
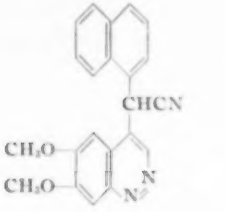
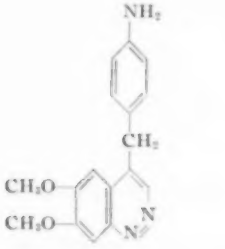
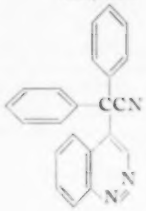
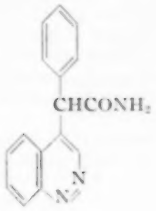
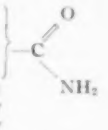
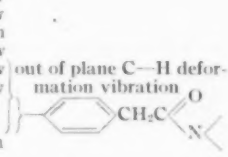
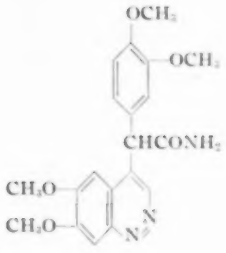
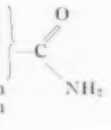
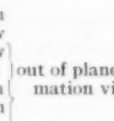
Compound	Wave Length in Microns					
	Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group		Miscellaneous
	4.65 w	6.35 m	6.25 w 6.70 m	7.80 w 7.94 m 8.07 m 8.28 m 8.52 m 8.62 m 8.81 m	11.40 w 11.70 m 12.46 m 13.00 m 13.35 m	out of plane C—H deformation vibration o-disubstituted phenyl
	4.65 m	6.38 s	6.16 m 6.25 w 6.68 s	7.87 m 8.00 m 8.16 m 8.27 m 8.35 m 8.97 m 9.20 m	11.52 m 11.93 m 12.05 w 12.32 w 12.55 m 13.15 w 14.25 w	out of plane C—H deformation vibration -mono substituted phenyl
	4.64 s	6.36 s	6.15 m 6.25 m 6.63 s	7.92 s 8.05 s 8.14 s 8.28 s 8.56 s 8.76 s 8.95 s 9.15 s 9.48 m	9.78 s 10.10 s 11.58 w 11.90 s 12.00 m 12.25 s 13.08 m 14.20 w	out of plane C—H deformation vibration o-disubstituted phenyl
	4.65 s	6.33 s	6.35 s 6.55 m (naphthyl)	7.63 m 7.87 s 7.95 m 8.08 m 8.25 s 8.58 m 8.82 m 9.24 m	9.52 s 9.85 m 9.98 s 11.39 m 11.82 m 12.08 m 12.45 m 12.80 s 13.38 m	out of plane C—H deformation vibration naphthyl
		6.35 w	6.50 w 6.60 m	7.65 m 7.90 m 8.10 m 8.27 m 8.57 m	6.21 m 9.65 m 9.75 m 10.12 m 11.37 w 11.88 m 12.00 m 12.87 w	aniline (or phenyl ?) out of plane C—H deformation vibration
	4.50 w	6.38 m	6.20 w 6.30 w 6.72 s		7.14 m 8.49 m 8.70 m 8.75 m 9.34 m 9.70 m 9.99 m	

TABLE I (*Continued*)

Compound	Wave Length in Microns: Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group		Miscellaneous
					11.03 w 11.22 w 12.20 w 12.40 w 12.60 w 13.17 s 13.38 s 14.05 m 14.21 s 14.32 s 14.70 m	out of plane C—H deformation vibration
		6.38 m	6.70 w		3.03 s 3.18 s 5.95 s 6.13 s 7.10 m 7.70 w 8.03 w 8.86 w 8.80 m 10.48 w 11.88 w 12.30 w 13.25 s 14.23 s 14.93 m	  out of plane C—H deformation vibration
		6.30 m	6.70 s	7.78 m 7.90 m 8.05 m 8.15 s 8.29 s 8.53 m 8.72 s	3.03 s 3.18 s 5.97 s 6.18 s 7.15 m 9.63 m 9.76 s 10.13 m 10.35 w 11.38 w 11.80 s 12.00 m 12.45 m 12.68 w 13.12 m 14.85 m	  out of plane C—H deformation vibration o-substituted phenyl

pressure. A yield of 2.76 Gm. (97%) of crude 4-aminocinnoline melting at 208–212° was obtained. After purification by solution in dilute hydrochloric acid, precipitation with dilute sodium hydroxide, and subsequent crystallization from aqueous ethanol (charcoal), there was obtained 2.1 Gm. (87%), of fine yellow crystals m. p. 215.5–218° (Keneford, Schofield, and Simpson (6) report 212–213°). The identity of the 4-aminocinnoline, prepared in this manner, was confirmed by the preparation of 4-acetylaminocinnoline. Seven-tenths of a gram of 4-aminocinnoline was refluxed for six and one-half hours with a mixture of 3 ml. of acetic anhydride and 8 ml. of dry pyridine. The amine was slowly soluble in the acetic anhydride-pyridine mixture. A part of the pyridine-acetic anhydride mixture was removed by vacuum evaporation and the residue was poured into cold water. The product which separated was purified by crystallization from ethanol (charcoal). The purified product amounted to 0.22 Gm. of fine crystals melting at 277° with decomposition.

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Studies on the Pharmacology of Cobalt Chloride*

By H. R. MURDOCK, Jr.

The studies reported show that at dosage levels approximating those used in therapeutics, cobalt is absorbed in the rat in significant quantity. Oral administration has been shown to produce demonstrable increases in erythropoietin in the blood and, as a result, peripheral blood values respond to an extent proportional to the dosage administered.

COBALT THERAPY is widely used in the treatment of certain forms of anemia, both in Europe and in the United States. However, its mechanism of action remained obscure prior to the demonstration by the Jacobson group (1) that its erythropoietic effect was due to increased erythropoietin formation.

Earlier pharmacologic studies dealing with absorption, excretion, toxicity, and metabolism of cobalt were based largely on parenteral administration. Since, however, oral use represents the principal therapeutic application, it was considered important to study these same factors upon oral administration and to relate these findings to previous work.

Our studies with oral cobalt administration form the basis of this report.

MATERIALS AND METHODS

Absorption and Excretion.—Radioactive cobalt chloride solution was given orally in single individual doses by metal stomach tube to fasted adult albino rats. The solutions were prepared with sufficient Co^{58} chloride so that each dose represented approximately 1 μc. of radioactivity; they were administered at dosage levels of 0.25 mg./Kg., 1.25 mg./Kg., 5 mg./Kg., and 10 mg./Kg. of elemental cobalt. The animals were immediately placed in metabolism cages and the pooled urine and feces collected separately from each group during a seventy-two-hour period. Three to six animals comprised each dosage level group.

The radioactivity was determined at intervals in aliquot portions of both urine and feces in a well-type scintillation counter. Feces were digested with concentrated hydrochloric acid prior to counting.

Determination of Erythropoietin.—We have modified the method of Goldwasser, *et al.* (1), to permit the oral administration of cobalt. Large fasted rats were given 100 mg./Kg. of elemental cobalt, as cobalt chloride, orally by metal stomach tube. They were exsanguinated twenty hours later, and the plasma obtained by centrifuging the heparinized blood. Two milliliters of this plasma was injected intraperitoneally into fasted rats weighing 120 to 150 Gm. for two successive days. On the third day, 1 to 2 μc. of Fe^{59} were injected into the external jugular. On the next succeeding day, blood was taken from the heart and the radioactivity counted in a well-type scintillation counter. The Fe^{59} utilization was calculated and compared with controls. Hemoglobin was also determined at the same time and rats with abnormal values were discarded.

Hemopoietic Studies.—Thirty adult male rats, 225 to 250 Gm., were given 10 mg./Kg. of elemental cobalt, as cobalt chloride, orally by metal stomach tube five days a week. A similar group of twenty-six rats received an equal volume of isotonic saline by metal stomach tube. All animals were maintained throughout the experiment on a standard diet and were kept in cages subject to identical conditions of temperature, etc.

The erythrocyte count, hemoglobin, and hematocrit determinations were done at frequent intervals during the one hundred fifty-day period of study. At the end of the study, all rats were autopsied and tissues taken for microscopic pathologic examination.

Blood for hematologic determinations was collected in heparinized vials from the tail of lightly etherized animals. Erythrocyte determinations were done by standard methods using a Spencer "Bright-Line" counting chamber. Hemoglobin was determined by the cyanmethemoglobin method. Hematocrit determinations were done using Van Allen tubes. Blood volume determinations were not considered necessary because of the well-established fact that cobalt administration has no significant effect on blood volume (2).

Toxicity.—The oral LD_{50} was determined by the usual methods in adult fasted rats. Ten rats were used at each of the six dosage levels.

In determining chronic toxicity, one group of eight rats weighing approximately 225 Gm. received 10 mg./Kg. of elemental cobalt orally, as cobalt chloride, five days a week by metal stomach tube for two hundred forty days. A second group of ten animals, weighing approximately 98 Gm., received 4 mg./Kg. of elemental cobalt in the same manner for two hundred ten days. At the conclusion of the test period, all animals were sacrificed, organs examined and weighed, and sections made for microscopic examination.

RESULTS

Absorption and Excretion.—Absorption was rapid as evidenced by the appearance of labeled cobalt within thirty minutes in the urine of all animals tested (Table I). The principal portion of the absorbed isotope was excreted in the urine during the first few hours after administration.

Of the total dose administered, approximately 27, 25, 15, and 12% was excreted in the urine at the dosage levels of 0.25, 1.25, 5, and 10 mg./Kg. of elemental cobalt, respectively. Since biliary excretion is also known to be a factor, these values probably represent minimum absorption figures.

Urinary excretion plotted against time on semilogarithmic paper appears to approach a straight-line relationship (Fig. 1). Cobalt, therefore, adminis-

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Technical assistance involved in these studies was given by E. W. Tryczynski and J. M. Wagner. Appreciation is extended to Dr. L. J. Klotz for assistance in preparing this manuscript.

TABLE I.—URINARY EXCRETION OF DIFFERENT DOSAGES OF Co^{58} AFTER ORAL ADMINISTRATION OF LABELED COBALT CHLORIDE TO RATS

Hours After Admin.	0.25 mg. Co/Kg., %	1.25 mg. Co/Kg., %	5 mg. Co/Kg., %	10 mg. Co/Kg., %
0.5	1.1	1.2	..	..
2.0	11.2	8.7	4.4	0.2
4.0	16.6	14.0	8.0	2.3
6.0	18.3	16.8	9.3	4.5
24.0	23.9	21.5	10.4	7.0
48.0	24.7	23.6	13.1	10.8
72.0	26.73	25.0	14.5	11.5

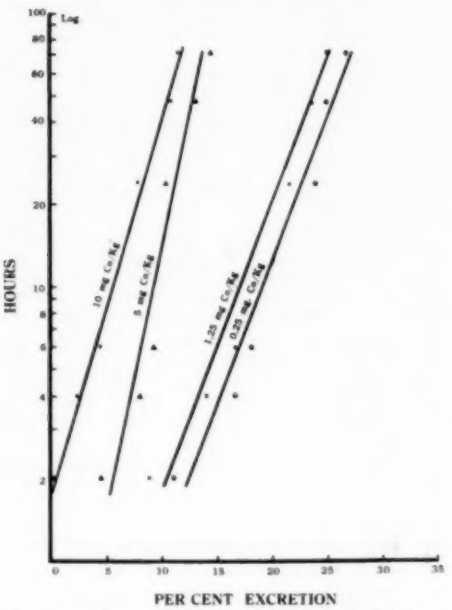

Fig. 1.—Urinary excretion of orally administered radiocobalt in rats. (Ordinate given in log-scale).

tered orally as the chloride, is absorbed in quantities roughly proportional to the dose given and is excreted principally in the urine.

Erythropoietin Determination.—Parenteral cobalt administration results in increased radioiron red cell incorporation due to erythropoietin formation (1). As an assay procedure, however, the method requires doses of cobalt near the LD_{50} level and mortality is high among the test animals.

Values obtained with orally administered cobalt at a dosage of 100 mg./Kg. were essentially similar to those found after parenteral administration, but the mortality was greatly decreased. There was no apparent inconsistency between results obtained by the two methods.

These findings, therefore, indicate both that oral cobalt is effective in enhancing the formation of erythropoietin and that the assay technique may advantageously be modified to use oral administration.

Results are shown in Table II.

Hemopoietic Response.—Results are shown in Fig. 2. The values represent the average of each group of animals. It is of interest to note that a

TABLE II.—Fe^{59} RED CELL INCORPORATION IN RATS TREATED WITH COBALT-ENHANCED ERYTHROPOIETIN-RICH PLASMA

Group	No. of Rats	% Fe^{59} Incorporation	S.D.
Controls	32	8.3	±3.2
Erythropoietin-rich plasma (from oral cobalt treatment)	29	19.3	±4.9
Erythropoietin-rich plasma (from subcutaneous cobalt treatment)	5	21.3	...

steady increase in the hematopoietic indexes occurred during the first sixty days and thereafter the polycythemia was maintained without further significant change. Since the blood indexes were essentially unchanged after ninety days, some of the rats were dropped from the experiment, leaving 16 rats in each group. Eleven animals in the control group and 12 cobalt-treated animals were carried throughout the experiment. Nine deaths occurred, mortality being due either to injury by the metal stomach tube or to anesthesia.

Table III shows the calculated mean corpuscular hemoglobin concentration and mean cell volume. It is evident that no significant changes occurred in these indexes and that, therefore, both cell size and hemoglobin content per cell, under cobalt therapy, remained approximately the same as in the normal animals.

These data are in keeping with the absorption data presented and indicate that oral cobalt is effective as a hemopoietic agent.

Toxicity.—The LD_{50} of cobalt chloride was found to be 144 mg. elemental cobalt per kilogram (Table IV). Toxic symptoms at the higher dosages appeared immediately after administration, and within six hours the rats either died or began to recover.

TABLE III.—HEMATOLOGIC INDEXES FOR COBALT-TREATED RATS

Days Treatment	Treated M.C.H.C., %	Treated M.C.V.	Controls M.C.H.C., %	Controls M.C.V.
0	36	54.6 cu. μ[a]	34	56.0 cu. μ[a]
30	37	54.0 cu. μ	35	55.3 cu. μ
60	38	53.6 cu. μ	39	52.5 cu. μ
90	37	53.4 cu. μ	39	...
150	38	52.9 cu. μ	37	50.5 cu. μ

[a] Cu. μ—cubic microns; M.C.H.C.—mean corpuscular hemoglobin concentration; M.C.V.—mean cell volume.

TABLE IV.—LD_{50} DATA FOR COBALT CHLORIDE ADMINISTERED ORALLY TO FASTED RATS

Mg. Co/Kg.	No. Deaths / No. Injected
98	1/10
122	2/10
137	3/10
150	5/10
153	7/10
191	10/10

LD_{50} = 144(127–162) mg. Co/Kg.[a]

[a] Method of Litchfield and Wilcoxon (3).

TABLE V.—BODY WEIGHTS AND RATIO OF ORGAN TO BODY WEIGHTS OF RATS RECEIVING ORAL COBALT CHLORIDE

Group	Daily Dose mg. Co/Kg.	Duration of Expt., Days	Body Weight, Gm. Initial	Body Weight, Gm. Final	Ratio of Organ Weight, % Adrenal × 100	Ratio of Organ Weight, % Kidney	Ratio of Organ Weight, % Liver	Ratio of Organ Weight, % Spleen
Control	.. (5)[a]	240	228	424	0.98	0.69	3.5	..
Cobalt chloride	10 (6)	240	224	382	0.98	0.75	3.8	..
Control	.. (7)	218	98	404	0.99	0.58	2.9	0.14
Cobalt chloride	4 (6)	218	98	396	1.00	0.67	3.4	0.20

[a] Number of animals in parenthesis.

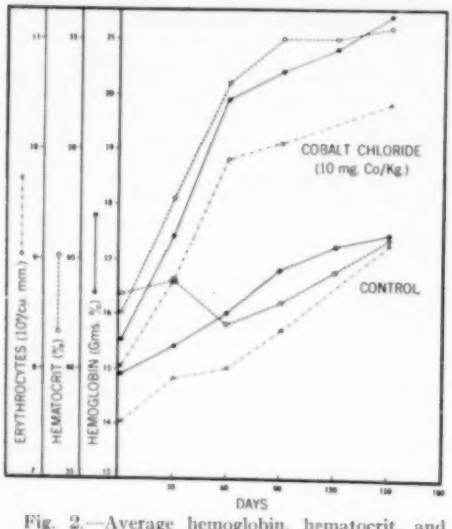

Fig. 2.—Average hemoglobin, hematocrit, and erythrocyte count of normal rats receiving 10 mg./Kg. elemental cobalt five days a week as compared with control animals.

Table V shows the initial and final body weights and ratio of organ weight to body weight of the rats receiving oral cobalt chronically. A few of the animals were lost due to injury caused by the daily stomach tubing. It was considered that there were no significant differences in the body weights of controls and the animals receiving cobalt. The adrenal weights were approximately the same but the average weights of kidneys, livers, and spleens of the cobalt-treated groups were slightly heavier than the controls. Extra-medullary erythropoiesis, a common phenomenon in rats, may be responsible for the increased weights of spleen and liver.

Microscopic examination of the kidneys of the rats on the 10 mg./Kg. dosage level, but not on the 4 mg. dose level, revealed some necrosis of the cells lining the tubules. This was not unexpected since the kidney is the main pathway for the excretion of cobalt (4). The phenomenon is reversible, however, since examination of the kidneys of rats autopsied thirty days after cobalt administration was discontinued showed no necrosis and were normal compared to kidneys from control rats. Similar changes have been described by others (5).

The bone marrow of the rats receiving cobalt was hypercellular, particularly in the cells of the erythroid series. The Malpighian corpuscles of the spleen were enlarged, as described by Hopps, *et al.* (6), in rats receiving subcutaneous injections of cobalt chloride. Both of these changes are in accord with the polycythemia produced in these rats.

DISCUSSION

Studies on the extent of cobalt absorption, after oral administration, have been contradictory. Administration in animal feed has failed to provide definitive limits of dosage and there has been no attempt to correlate findings with clinically used dosage levels.

Minute doses are said to be completely absorbed (7) and larger dosages half absorbed (8), but others have reported insignificant gastrointestinal absorption (9). Therapeutically, adult dosage levels approximate 0.25 mg./Kg. of elemental cobalt, as cobalt chloride, per day. At this level, in our studies, absorption approximated 25% of the dose administered. Urinary excretion of the absorbed cobalt was relatively complete within twenty-four hours. These data are in accord with the findings of others (10).

Absorption after oral administration is sufficiently great and consistent to permit the demonstration of increased erythropoietin formation by cobalt. The consequence is a marked parallel increase in hemoglobin, red cell count, and hematocrit while the red cell indexes remain nearly constant and do not differ appreciably from those of controls. Continued administration eventually results in a plateau for hemoglobin and hematocrit values, with the polycythemia being maintained at the new level. The response is proportional to the dosage used.

Acute toxicity studies indicate that cobalt chloride, given orally, is not very toxic and long-term administration indicates that at dosage levels approximating 10 times those used in therapy, no significant toxic effects are present. As has previously been reported (5), massive experimental doses may cause transient kidney damage.

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A Pharmacologic Evaluation of Certain Cobalt-Containing Hemopoietic Agents*

By H. R. MURDOCK, Jr., and L. J. KLOTZ*

Gastrointestinal absorption of cobalt-containing compounds appears to depend, in part, on a mechanism which concerns cobalt alone and is not dependent on the remainder of the molecule. The systemic toxicity appears to increase directly with the dissociation to supply cobalt ion. Cobalt disodium ethylenediamine tetra-acetate shows little dissociation and little systemic toxicity. The enhancement of erythropoietin formation can be accomplished in approximately the same degree by equivalent cobalt doses given as ionic cobalt, complex-ion, or difficultly dissociated chelated cobalt. This is subsequently reflected in peripheral blood indexes. Both cobalt polyglucopyranose and cobalt disodium ethylenediamine tetra-acetate have higher therapeutic indexes, as determined in the rat, than does cobalt chloride. Both would appear suitable for clinical trial. No evidence of chronic toxicity was found from prolonged oral administration of cobalt polyglucopyranose or cobalt disodium ethylenediamine tetra-acetate in the rat. Cobalt disodium ethylenediamine tetra-acetate (Copoietin A) appears to represent the most satisfactory type of cobalt compound tested and should be worthy of clinical trial as a therapeutic agent.

ALTHOUGH cobaltous chloride has received wide acceptance as a therapeutic agent in the treatment of anemia, its usefulness may be limited by gastrointestinal intolerance which often appears above dosage levels of about 100 mg. per day in humans. Furthermore, the "heavy metal effect" and the local irritant action of cobalt chloride practically preclude the use of effective doses by parenteral administration.

Numerous other cobalt compounds, usually of the "complex" type, have been described in the literature as having therapeutic merit because of decreased toxicity, decreased side effects, or practicality for parenteral use. None has found general medical acceptance at this time.

It is the purpose of this report to describe some of the pharmacologic properties of certain cobalt compounds which appear to offer advantages for possible therapeutic use. Three compounds studied were: cobalt-niacinamide complex, cobalt polyglucopyranose, and cobalt disodium ethylenediamine tetra-acetate.

MATERIALS AND METHODS

Preparation of Cobalt Compounds.—Cobalt-niacinamide complex was prepared according to published methods (1). It is a red crystalline material, soluble in water and alcohol, and contains about 15% elemental cobalt.

Cobalt polyglucopyranose is a complex prepared in this laboratory as follows. Five grams of "soluble starch" and 1 Gm. of cobaltous chloride in distilled water is brought to approximately pH 11 by the addition of 10% aqueous sodium hydroxide. The formation of the complex is indicated by the appearance of a deep blue color. Excess isopropanol (one to three volumes) is then added. The complex precipitates and is washed free of alkaline chloride and any unreacted cobaltous chloride by washing with several portions of isopropyl alcohol. It appears that the cobalt atom is linked to the 1,4 oxygen bridge of the polyglucopyranose structure. It is greenish blue, noncrystalline solid, slightly soluble in water, insoluble in alcohol, and containing about 7% elemental cobalt.

The cobalt disodium ethylenediamine tetra-acetate chelate was prepared according to the method described by Schwarzenbach (2). It is a red crystalline material containing about 15% elemental cobalt which forms stable solutions in water and alcohol. Cobalt chloride used in certain of these studies was the commercial C. P. cobalt chloride hexahydrate with the purity verified by chemical assay

Toxicity Studies.—Adult albino rats were used for the LD_{50} determinations. They were fasted overnight and the compound given the next morning so that the animals could be closely observed during the day. Ten rats were used at each dosage and at least five dosage levels were used for each compound.

Chronic toxicity studies were conducted on both weanling and adult rats. The cobalt compounds were administered orally five days a week by metal stomach tube. At the end of the study the rats were autopsied and the tissues and organs examined for gross as well as microscopic changes. Two groups of rats kept under identical conditions were given cobalt chloride and saline, respectively, as controls.

Absorption and Excretion Studies.—Labeled radiocobalt (Co^{58}) compounds were specially prepared for these studies. The method of preparation of the compounds used was that described above except that proper amounts of radiocobalt (Co^{58}) were used along with nonradioactive cobalt in the synthesis. The radioactive compound was given orally to fasted rats by metal stomach tube in a dosage representing 0.25 and 1.25 mg./Kg. of elemental cobalt. The lower dose closely approximates the common daily adult clinical dose of 60 mg. of cobalt chloride calculated on a weight basis. The rats were immediately placed in metabolism cages and the urine and

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Technical assistance involved in these studies was given by Joseph M. Wagner, Emil W. Tryczynski, and Joseph B. Vaughan.

feces collected separately. Aliquot portions of the urine and of the acid-digested feces were counted in a well-type scintillation counter and the total amount of excreted cobalt was calculated.

Determination of Erythropoietin.—The procedure consisted of giving the cobalt compound orally to large eighteen-hour fasted rats at a dosage representing 100 mg./Kg. of elemental cobalt. Eighteen to twenty hours later the animals were exsanguinated under light ether anesthesia. Two milliliters of plasma from this blood was injected intraperitoneally into eighteen-hour fasted rats which were deprived of food throughout the assay. Twenty-four hours later, these animals received another 2-ml. injection of plasma. On the third day they were injected intravenously with 1 to 2 μc. of radioiron (Fe^{59}). Twenty-four hours later, blood was obtained from the heart, the Fe^{59} activity determined, and the percentage of erythrocyte incorporation of radioiron calculated. Details of the method are published elsewhere (3).

Hemopoietic Studies.—The methods used were similar to those reported previously (4). The compounds were administered five days a week to adult male rats by metal stomach tube. Hematologic data were determined at intervals. Blood samples were taken from the tail vein observing the usual precautions to avoid error. Erythrocyte counts were carried out using a Spencer "Bright-line" counting chamber; hemoglobin was determined photocolorimetrically; and hematocrit by the Van Allen method.

Urinary Calcium.—Because of certain theoretical considerations, it was thought desirable to determine the effect of one of our compounds (cobalt disodium ethylenediamine tetra-acetate) on urinary calcium excretion. For this purpose, total urinary calcium was determined by a modification of the method of Shohl and Pedley (5) in five human subjects. The values obtained on each subject during a control period of four days were taken as normal. This was followed by a four-day period during which time each subject took 440 mg. a day of cobalt disodium ethylenediamine tetra-acetate in divided doses. This dose represents somewhat more than four times the elemental cobalt dosage usually given as cobalt chloride. Diet was not restricted except that the subjects attempted to eat and drink roughly the same amount of high calcium foods each day they participated in the study.

RESULTS

Acute Toxicity.—The acute LD_{50} for cobalt chloride and for each of the three cobalt compounds, cobalt-niacinamide, cobalt polyglucopyranose, and cobalt disodium ethylenediamine tetra-acetate in the rat are given in Table I. Data are given in mg./Kg. of elemental cobalt represented by the dosage of the compound administered. Cobalt-niacinamide is of the same order of acute toxicity as cobalt chloride and in this respect does not appear to offer any advantages over the latter.

The intraperitoneal LD_{50} of cobalt polyglucopyranose represents 26 mg./Kg. of elemental cobalt. This is less than one-third as toxic as cobalt chloride. The onset of toxic symptoms at the higher dosages of cobalt polyglucopyranose appeared much later and were less severe than the symptoms following cobalt chloride injections. The rats that died following cobalt chloride injection usually did so within six hours; following cobalt polyglucopyranose, the majority of deaths occurred between twelve and twenty-four hours.

TABLE I.—ACUTE LD_{50} OF COBALT COMPOUNDS IN THE RAT

Compound	LD_{50} in mg./Kg. Elemental Cobalt	
	Intraperitoneal	Oral
Cobalt chloride	7.9 (7.4–8.5)[a]	144 (127–162)[a]
Cobalt-niacinamide	.....	134 (121–149)[a]
Cobalt polyglucopyranose	26.0 (1.96–35.1)[a]	>328
Cobalt disodium ethylenediamine tetra-acetate	>800	>1000

[a] Method of Litchfield and Wilcoxon (6).

It was not possible to obtain an exact oral LD_{50} with cobalt polyglucopyranose in the rat. Neither deaths nor serious toxic symptoms occurred at dosages representing less than 328 mg./Kg. of elemental cobalt. At this dosage, no immediate toxic symptoms were seen except for a black diarrhea appearing on the day following administration. However, two of the ten rats died on the third day. Autopsy of a rat which appeared quite ill after receiving this dosage revealed no abnormality other than hyperemia of the gastrointestinal tract. It was impractical to give higher dosages because of the relatively low solubility of the compound.

It was not possible to obtain either an oral or intraperitoneal LD_{50} in the rat with cobalt disodium ethylenediamine tetra-acetate. Orally, dosages representing as high as 1,000 mg./Kg. of elemental cobalt produced only mild toxic symptoms as did intraperitoneal injections containing up to 800 mg./Kg. of elemental cobalt. All the rats seemed to be fully recovered after twenty-four hours and none died. It was impractical to give higher dosages because of the large volume of even highly concentrated solution which was involved.

In comparison, the oral LD_{50} of cobalt chloride represents 144 mg./Kg. of elemental cobalt. The toxic symptoms appeared early and death usually occurred within six hours.

Rabbits injected intravenously with 25 mg./Kg. elemental cobalt as cobalt chloride invariably went into deep shock. The blood pressure fall was so severe that no bleeding took place at the site of injection.

Rabbits receiving twice this dose of elemental cobalt intravenously, given as cobalt disodium ethylenediamine tetra-acetate, showed no detectable toxic symptoms. Four times this dose (approximately 100 mg./Kg. elemental cobalt), administered as rapidly as possible, still produced no toxic symptoms except for a questionable increase in respiratory rate. There were no signs of shock and the rabbits remained alert and active.

Chronic Toxicity.—The results obtained from prolonged oral administration of the three cobalt compounds in the rat are shown in Table II. Data for the number of rats in each group refer to the number of rats autopsied at the conclusion of the experiment

TABLE II.—AVERAGE BODY AND ORGAN WEIGHT OF RATS RECEIVING COBALT COMPOUNDS ORALLY

Compound Administered	Dose mg./Kg., Co	No. Days on Expt.	Initial Body Wt., Gm.	Final Body Wt., Gm.	Ratio of Organ Wt. to Body Wt., % Spleen	Adrenal × 100	Kidney	Liver
Cobalt PGP[a]	4 (8)[b]	240	92	356	0.15	1.0	0.55	3.1
Cobalt PGP	8 (8)	240	93	385	0.13	1.1	0.54	2.9
Cobalt EDTA[c]	4 (8)	180	98	366	0.22	1.2	0.66	3.5
Cobalt EDTA	8 (8)	180	98	398	0.18	1.0	0.66	3.1
Cobalt chloride	4 (6)	218	98	396	0.20	1.0	0.67	3.4
Saline	.. (7)	218	98	404	0.14	0.96	0.62	2.9
Cobalt EDTA	10 (8)	240	239	501	..	0.98	0.66	3.2
Cobalt EDTA	20 (6)	240	194	424	..	0.95	0.71	3.4
Cobalt EDTA	40 (6)	240	233	436	..	0.92	0.73	3.7
Cobalt chloride	10 (6)	240	224	382	..	0.98	0.75	3.8
Saline	.. (5)	240	228	464	..	0.98	0.68	3.5

[a] Cobalt polyglucopyranose. [b] Number of animals autopsied. [c] Cobalt disodium ethylenediamine tetra-acetate.

since an occasional animal died due to injury caused by use of the metal stomach tube.

Administration of cobalt polyglucopyranose in a dosage representing 4 and 8 mg./Kg. of elemental cobalt to adult rats for two hundred forty days had no effect on the weight gain. At autopsy, no gross or microscopic pathologic findings were present. The ratio of the spleen, liver, and adrenal weights to the body weight were similar to the values obtained for the other groups. Values obtained for the kidney-body weight ratios were somewhat lower but the range overlapped values obtained with rats receiving cobalt chloride.

Cobalt disodium ethylenediamine tetra-acetate in a dosage of 4 and 8 mg./Kg. of elemental cobalt had no effect on the weight gain of weanling rats. The ratio of the organ to body weights were within normal range and no gross or microscopic pathologic findings were present at autopsy.

Ten, 20, and 40 mg./Kg. of elemental cobalt given as cobalt disodium ethylenediamine tetra-acetate also had no effect on the weight gain of adult rats when administered for two hundred forty days. At autopsy there were no gross or microscopic pathologic findings which could be attributed to the chelate. The organ to body weight ratios were also within normal range.

In contrast, continued oral administration of 40 mg./Kg. of cobalt as cobalt chloride causes severe anorexia and other toxic symptoms.

It is of interest to note that microscopic examination of the kidneys of those rats receiving 10 mg./Kg. of cobalt, as cobalt chloride, showed evidence of degeneration of the cells lining the tubules. This had previously been reported (7).

It was concluded that neither cobalt disodium ethylenediamine tetra-acetate nor cobalt polyglucopyranose showed evidence of toxicity when administered orally in the above dosages.

It has been shown that about 15 to 35% of the cobalt administered parenterally as cobalt disodium ethylenediamine tetra-acetate is excreted as ionic cobalt (8). It follows, therefore, that a portion of the dose given probably frees a small amount of disodium ethylenediamine tetra-acetic acid in the body. Previous work (9) has shown that this substance may chelate with and thus remove calcium from the body and that, in large intravenous doses, calcium deprivation and even tetany may result.

The usual human oral dosage level of about 400 mg. of cobalt disodium ethylenediamine tetra-acetate per day would provide 340 mg. of the disodium chelating agent, assuming complete dissociation of the cobalt chelate. This quantity could remove a maximum of about 40 mg. of calcium from the body per day.

Calcium excretion values for human subjects before and during daily oral administration of 440 mg. of cobalt disodium ethylenediamine tetra-acetate are given in Fig. 1. The twenty-four-hour urinary calcium varied greatly from subject to subject. One subject, E. T., with a past history of kidney stones, showed high values. The other subjects had no history of kidney or urinary pathology.

It is evident that there was no significant difference in the total calcium excretion during the experimental period from that during the control period and that administration of cobalt disodium ethylenediamine tetra-acetate does not significantly promote the excretion of calcium in the doses used.

Absorption and Excretion.—Since the lack of systemic toxicity of cobalt disodium ethylenediamine tetra-acetate and cobalt polyglucopyranose could be due to decreased absorption, it seemed desirable to determine whether the cobalt from these compounds was actually absorbed. Gastrointestinal absorption was estimated by preparing the two compounds with labeled radiocobalt and determining the urinary excretion of the radiocobalt following oral administration.

It is recognized that this is not a quantitative measure of absorption since cobalt is also excreted in the bile. The technique does, however, furnish evidence of absorption and provides useful data concerning the relative amounts absorbed.

Single oral doses of labeled radiocobalt chloride, cobalt polyglucopyranose, and cobalt disodium ethylenediamine tetra-acetate were given to represent 0.25 mg./Kg. or 1.25 mg./Kg. of elemental cobalt in each instance. Results are shown in Table III.

There appears to be little difference in excretion, and therefore probably in absorption, among the three compounds at the lower dosage level. Some decreases in absorption, however, appear to be present at the higher dosage. With both cobalt disodium ethylenediamine tetra-acetate and cobalt polyglucopyranose, radiocobalt was found in the urine within half an hour, thus indicating rapid absorption. Over 95% of the cobalt excreted in the urine was recovered within twenty-four hours, indicating rapid absorption and excretion. When the urinary excretion values were plotted against time on semilog paper, a straight line was obtained. This is

TABLE III.—URINARY EXCRETION OF Co^{58} AS PER CENT OF ORAL DOSE

Hrs. After Dose	0.25 mg./Kg. Co, %			1.25 mg./Kg. Co, %		
	CoEDTA[a]	CoPGP[b]	$CoCl_2$	CoEDTA	CoPGP	$CoCl_2$
0.5	0.9	0.1	1.1	0.1	0.3	1.2
2.5	12.9	11.3	14.1	4.4	2.8	13.2
4.5	16.0	18.8	16.6	8.8	8.6	14.0
6.5	18.9	22.6	18.3	9.7	9.6	16.8
24.0	26.9	24.4	24.8	15.9	12.3	21.5
48.0	28.2	25.9	..	16.9	12.8	..
72.0	28.3	27.0	..	17.3	13.1	..
96.0	28.3	27.1	26.7	17.5	13.2	25.0

[a] Cobalt disodium ethylenediamine tetra-acetate. [b] Cobalt polyglucopyranose.

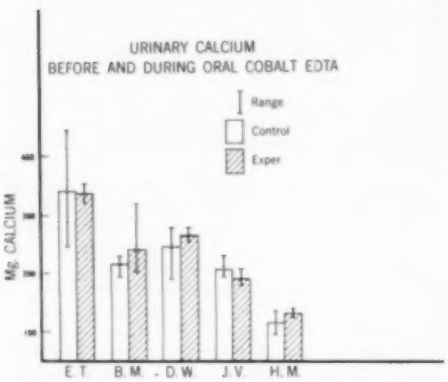

Fig. 1.—Urinary calcium excretion in human subjects before and during oral administration of cobalt disodium ethylenediamine tetra-acetate. Each bar represents the average for four days.

taken as indicative of little or no retention of cobalt in the body.

Erythropoietin Production.—Since cobalt is absorbed when given in the form of these compounds, its physiologic availability from a nonionizing chelate such as cobalt disodium ethylenediamine tetra-acetate was of interest. As a measure of availability of the cobalt component, the effect on the production of erythropoietin, the erythropoietic hormone, was determined.

Results are given in Table IV. They indicate that erythropoietin production was increased to approximately the same degree following cobalt disodium ethylenediamine tetra-acetate, cobalt polyglucopyranose, and cobalt chloride administration.

Hemopoietic Effect.—To determine whether these erythropoietin increases would be reflected in increased peripheral blood values, the ability of these compounds to produce the characteristic polycythemia in rats was studied. This method has previously been used in evaluating the hemopoietic effect of other cobalt compounds (4).

TABLE IV.—Fe^{59} ERYTHROCYTE INCORPORATION FROM ERYTHROPOIETIN-RICH SERUM PRODUCED BY COBALT ADMINISTRATION

Group	No. Rats	Av. Incorporation, %
Controls	18	8.3
From cobalt chloride treatment	18	19.7
From cobalt polyglucopyranose treatment	13	15.0
From cobalt disodium ethylenediamine tetra-acetate treatment	10	17.8

Oral administration of 10 mg./Kg. of elemental cobalt as cobalt polyglucopyranose, as cobalt-niacinamide, or as cobalt chloride produced equivalent increases in all three blood indexes as indicated in Table V. The hemopoietic effect of the two new cobalt compounds is, therefore, equivalent to that of cobalt chloride.

Cobalt disodium ethylenediamine tetra-acetate was administered orally in dosages of 10, 20, and 40 mg./Kg. of elemental cobalt (Table V). The 10 and 20 mg./Kg. dosages did not have any significant effect on the blood indexes. The 40 mg./Kg. dose, however, was equally as effective as cobalt chloride at the 10 mg./Kg. elemental cobalt dose level. This study was continued for five additional months with no further significant increases in the blood indexes.

Both cobalt-niacinamide and cobalt polyglucopyranose were equivalent to cobalt chloride in increasing the blood indexes. Cobalt disodium ethylenediamine tetra-acetate is fully as effective in higher dosages and in view of its lesser toxicity, it has the greatest therapeutic index of any of the cobalt compounds studied.

DISCUSSION

Previous studies (3) and data presented in this report show that about 25% of the cobalt present in cobalt chloride is absorbed when given orally to rats at a dosage of 0.25 mg./Kg. elemental cobalt. This dose closely approximates, on a weight basis, the usual human dose. Using labeled (Co^{58}) cobalt polyglucopyranose or labeled (Co^{58}) cobalt disodium ethylenediamine tetra-acetate, at the same cobalt dosage level, similar gastrointestinal absorption percentages were evident, as estimated from urinary excretion data.

Chemical tests, *in vitro*, indicate that cobalt polyglucopyranose supplies cobaltous ion in the acid gastric juice but that the complex ion reforms in the alkaline intestinal fluids. The decreased toxicity of this compound argues that absorption is, at least in part, as the complex ion or total molecule rather than as ionic cobalt.

On the other hand, *in vitro* chemical tests indicate that the tightly bound chelate, cobalt disodium ethylenediamine tetra-acetate, does not dissociate to liberate free cobalt ions either in acid gastric juice or in alkaline intestinal fluids. It seems probable, therefore, that absorption is that of the undissociated molecule.

TABLE V.—HEMATOLOGIC RESPONSE IN ADULT MALE RATS RECEIVING COBALT COMPOUNDS ORALLY

Compound	Dose mg./Kg. Co	No. Days on Expt.	Erythrocytes million/cu. mm. Initial	Erythrocytes million/cu. mm. Final	Hemoglobin Gm./100 ml. Initial	Hemoglobin Gm./100 ml. Final	Hematocrit, % Initial	Hematocrit, % Final
Cobalt-niacinamide	10	60 (10)[a]	8.7	10.4	14.8	20.6	41.8	54.9
Cobalt chloride	10	60 (9)	8.2	9.9	15.1	21.2	40.4	53.6
Saline	..	60 (4)	7.1	6.9	13.8	15.7	38.5	41.5
Cobalt PGP[b]	10	55 (8)	7.8	9.9	14.9	18.5	41.2	52.1
Cobalt chloride	10	55 (4)	7.8	10.3	14.7	18.9	41.3	53.3
Saline	..	44 (4)	8.0	8.2	15.6	15.9	41.6	41.3
Cobalt EDTA[c]	10	55 (8)	7.4	8.3	14.6	17.9	39.9	46.8
Cobalt EDTA	20	55 (6)	7.2	8.8	15.7	19.1	40.8	48.5
Cobalt EDTA	40	55 (6)	7.1	9.1	14.8	19.5	39.9	51.0
Cobalt chloride	10	55 (6)	7.2	9.5	15.2	20.6	40.1	53.4
Saline	..	55 (5)	7.5	8.3	15.2	17.3	39.9	43.7

[a] Number of animals given in parenthesis. [b] Cobalt polyglucopyranose. [c] Cobalt disodium ethylenediamine tetra-acetate.

The similarity of absorption values with free cobalt ion from cobalt chloride, with cobalt complex ion from cobalt polyglucopyranose and with molecular cobalt disodium ethylenediamine tetra-acetate suggests that the absorption mechanism is dependent upon the cobalt atom rather than on the chemical nature of the remainder of the molecule.

It has been reported that ethylenediamine tetra-acetic acid and its alkali salts are poorly absorbed when given orally. Furthermore, the powerful chelating effect of this acidic structure could be expected to bind heavy metal such as cobalt, lead, iron, etc., and carry them unchanged through the gastrointestinal tract. In fact, an excess of this chelating agent appears to act in this fashion. For example, Child (10) reported a decrease in the hemopoietic action of cobalt chloride when administered orally with large dosages of disodium ethylene diamine tetra-acetate in the proportion of 1 to 2 and 1 to 50, and similar findings were obtained by Post (11) after concomitant intraperitoneal and subcutaneous injections of cobalt sulfate and calcium ethylenediamine tetra-acetate, respectively.

On the other hand, it has been shown (12) that the ethylenediamine tetra-acetate chelate compounds of lead, barium, and other similar heavy metals are absorbed from the gastrointestinal tract and our data indicate no inhibition of absorption of the cobalt compound itself.

Variation in the toxicity of cobalt compounds appears to be related, at least in part, to the degree of dissociation of the compound to furnish cobalt ion. Thus, freely ionizing cobalt chloride shows an LD_{50} representing 144 mg./Kg. elemental cobalt; the complex ion from cobalt polyglucopyranose shows a value of more than 328 mg./Kg.; the difficultly dissociated cobalt disodium ethylenediamine tetra-acetate gives a value greater than 1,000 mg./Kg. elemental cobalt.

The dissociation of these three compounds can be readily shown to occur in this same order by the addition of ammonium sulfide to their solutions. Precipitation occurs immediately in a solution of the freely ionized cobalt chloride; it is somewhat delayed in solutions of the polyglucopyranose compound and does not occur for twelve hours or longer in solutions of cobalt disodium ethylenediamine tetra-acetate.

As might be expected, the effect of parenteral injection of these compounds appears to follow the same order. Cobalt chloride injections must be carried out very slowly to avoid the systemic "heavy metal effect." Cobalt polyglucopyranose injections may be carried out more rapidly and in greater dosages without toxic effects. Cobalt disodium ethylenediamine tetra-acetate does not produce "heavy metal effects" upon intravenous injection in rabbits even when relatively large dosages are injected.

After absorption, cobalt compounds must either be effective *per se* or must liberate cobalt ion. It has been shown that approximately 15 to 35% of a given dose of cobalt disodium ethylenediamine tetra-acetate appears in the urine as ionic cobalt (8). Since the dissociation constant of this substance shows the cobalt to be very tightly bound, it would be expected that other compounds with greater dissociation constants would be more rapidly and completely broken down. The erythropoietin assay data reported above indicate that significantly greater potency cannot be obtained from a less tightly bound chelate or from a freely ionizing salt since cobalt disodium ethylenediamine tetra-acetate appears to be fully as potent as the less tightly bound compounds. The much lower toxicity of cobalt disodium ethylenediamine tetra-acetate therefore represents a significant improvement in the therapeutic index.

Clinical studies now in progress[1] tend to verify this conclusion and also indicate a marked diminution in gastrointestinal intolerance. The latter might be expected due to the absence of free cobalt ion in the gastric content.

Since cobalt ion is found in the urine after administration of cobalt disodium ethylenediamine tetra-acetate, it is probable that small quantities of ethylenediamine tetra-acetic acid or its disodium salt are formed. Thus, the possibility of toxicity from the latter substances must be considered. Ethylenediamine tetra-acetic acid and its salts do not appear to be very toxic although a few isolated cases of kidney damage have been reported after intravenous injection of large dosages (9). Dosages reported to cause this toxic effect are of the order of 90 mg./Kg. body weight. The administration of cobalt disodium ethylenediamine tetra-acetate in humans would approximate only 6 mg./Kg. a day of the chelate at a dosage of 0.5 Gm. This dosage, even assuming complete dissociation, could provide quantities of the ethylenediamine tetra-acetic acid compound far below the dosage at which toxic symptoms have been reported. In fact, the ethylenediamine tetra-acetic acid salts are generally regarded as relatively nontoxic (13).

[1] For clinical purposes, cobalt polyglucopyranose is identified as Copoietin PGP, cobalt disodium ethylenediamine tetra-acetate is identified as Copoietin A.

Since a portion of the cobalt administered as cobalt disodium ethylenediamine tetra-acetate can be recovered in the urine in ionic form, it was considered desirable to determine whether the free ethylenediamine tetra-acetate formed would chelate with serum calcium and result in calcium deprivation. That this dissociated ethylenediamine tetra-acetate in the blood has no significant effect on calcium excretion is evident from the data obtained. The unlikelihood of such an occurrence is evident, of course, when it is noted that ordinary dietary variation could easily cause decrease in calcium intake greater than the theoretical maximum calcium removal, even assuming complete dissociation of the cobalt chelate at the dosage used. Our animal studies, especially those on young growing rats, indicate no deleterious effect on either the kidney or other organs. In fact, the effect on the kidney appears to be even less significant than that seen following long continued administration of equally high dosages of elemental cobalt administered as cobalt chloride.

The three types of cobalt compounds tested appear to be similar in their ability to enhance the formation of erythropoietin in the rat and this increase in the erythropoietic hormone is reflected in a subsequent increase in peripheral blood values. Although larger doses of the chelate were required to produce equivalent hematologic increase, the decreased toxicity of this compound gives a much improved therapeutic index even at the necessary increased dosage level. These facts suggest that temporary high levels of cobalt ion are the cause of cobalt toxicity and that the lower, more prolonged levels given by the slowly dissociating chelate at these large experimental doses, although effective, are much less toxic.

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Dinitrile Formation in the Attempted Acylation of Malonamide and Its Alkyl Derivatives*

By KENNETH J. LISKA and ARVIND P. SHROFF

Attempted acylation of malonamide and mono-alkylmalonamides with acetyl chloride and pyridine indicates that no acylation takes place at room temperature. At elevated temperatures, the predominant reaction is dehydration to the corresponding dinitrile. Only one compound, *n*-butylmalonamide, gave a significant yield of acylated product, identified as N-acetyl-α-cyanocaproamide.

MALONAMIDE and its mono-alkyl derivatives offer two potential sites for the introduction of an acyl group. Treatment of these compounds with the usual reagents might theoretically lead to carbon-acylated products, or nitrogen-acylated products, or both. Liska (1), for example, acylated (2-hydroxy-1-naphthylmethyl)-malonamide with acetyl chloride and pyridine and showed that the product was a diacylated derivative, the second acetyl group having entered at either the methine carbon atom or the amide nitrogen atom.

It appears that no attempts have been made to acylate malonamide or mono-alkylmalonamides with acetyl chloride or other organic acyl halides. Boon and co-workers (2) have acylated C-ethyl-C-phenyl malonamide with acetyl chloride, in a sealed tube, to give an N,N′-diacylated product. Thompson (3) showed that α-chloroacetamide and α,α-dichloroacetamide failed to give any acylated product with different acid chlorides; dehydration was the only observed reaction, indicating that amides which possess electron withdrawing groups on an alpha carbon and which are relatively acidic (compared to acetamide) are less amenable to acylation of any kind and probably more easily dehydrated.

It was the purpose of this investigation to study the behavior of malonamide and certain of its alkyl derivatives when treated with acetyl chloride and pyridine, and to determine, if possible, the preferred site of acylation. Successful completion of this problem might lead to the solution of specific problems involving similar acylations.

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Abstracted from a thesis submitted to the Faculty of the Graduate School of Duquesne University by A. P. Shroff in partial fulfillment of the requirements for the degree of Master of Science.

EXPERIMENTAL

The following experiment typifies those acylations attempted at a temperature intermediate between room and reflux temperature.

Formation of Ethylmalondinitrile and Ammonium

Chloride in the Attempted Acylation of Ethylmalonamide.—Five grams (0.038 mole) of ethylmalonamide, 25 ml. of pyridine, and 2.9 ml. (0.038 mole) of acetyl chloride were heated in a 100-ml. round-bottom flask for one hour at 78–80°. Excess of pyridine was removed and the solid residue was taken up with ethyl alcohol. Filtration followed by recrystallization from 95% acetic acid removed 1.0 Gm. of ammonium chloride. The filtrate, on further distillation, gave ethylmalondinitrile, b. p. 208–209/760 mm. (Lit. 208°/756 mm. (4)). The yield was 2.3 ml. (61%).

Anal.[1]—Calcd. for $C_5H_6N_2$: N, 29.77; Found: N, 29.63.

Acylation of *n*-Butylmalonamide at Reflux Temperature.—Five grams (0.031 mole) of *n*-butylmalonamide contained in a 100-ml. round-bottom flask was dissolved with the aid of heat in 35 ml. of pyridine; 7.2 ml. (0.093 mole) of acetyl chloride was added dropwise to the solution. The solution was heated at reflux temperature for one hour. Precipitation of the acylated derivative was accomplished by concentrating the reaction solution to a volume of 8 ml., chilling, and adding water. Filtration gave a crude product which was recrystallized from water to yield 0.5 Gm. (9%) of white crystals of N-acetyl-α-cyanocaproamide, m. p. 116–117°. The infrared spectrum of the compound in Nujol showed a carbonyl band at 1653 cm.$^{-1}$ and a weak nitrile band at 2203 cm.$^{-1}$. There was no absorption in the ultraviolet.

Anal.[1]—Calcd. for $C_9H_{14}N_2O_2$: C, 59.3, H, 7.7, N, 15.38; Found: C, 59.2, H, 8.7, N, 15.33.

The filtrate on further distillation yielded a yellow liquid, *n*-butylmalondinitrile, b. p. 222–223/760 mm. The yield was 2.6 ml. (62%).

Anal.[2]—Calcd. for $C_7H_{10}N_2$: C, 68.85, H, 8.19, N, 22.95; Found: C, 68.93, H, 8.38, N, 22.85.

Degradation of N-Acetyl-α-cyanocaproamide to Caproamide.—Five hundred milligrams of N-acetyl-α-cyanocaproamide was heated in a 15-ml. round-bottom flask for one hour with 5 ml. of 10% sodium hydroxide solution. The ammonia evolved in the process was detected both by its odor and by litmus paper. Acidification of the solution with hydrochloric acid and chilling gave *n*-butylmalonic acid, m. p. 101–102° (Lit. 101.5–102° (5)). Decarboxylation of the *n*-butylmalonic acid was accomplished by refluxing in 5% hydrochloric acid solution for six hours. A yellowish liquid, presumably caproic acid, was extracted with ether. Four milliliters of thionyl chloride was added to the yellow liquid residue remaining after the removal of the ether and the mixture was refluxed for forty-five minutes. This solution was then added to 40 ml. of aqueous ammonia. Crystals of caproamide separated immediately. Filtration and recrystallization from water yielded pure caproamide, m. p. 101–102°. The mixed m. p. of *n*-butylmalonic acid and caproamide was 99–100°.

[1] Carbon and hydrogen analysis performed by Weiler and Strauss Microanalytical Laboratory, Oxford, England.

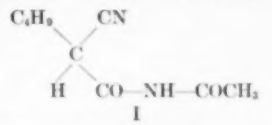
[2] Carbon and hydrogen analysis performed by Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y.

DISCUSSION

The initial acylation reaction for malonamide, ethylmalonamide, propylmalonamide, and butylmalonamide was attempted using acetyl chloride and pyridine at room temperature. Unchanged starting material was recovered in quantitative yield from each reaction. Varying the molar ratio of the amides to acylating agent did not change the results in any case.

At a temperature between room temperature and reflux temperature, each amide mentioned above underwent hydrolysis and dehydration when treated with acetyl chloride and pyridine. The hydrolysis product was ammonium chloride, the yields of which ranged from 19 to 30%; the dehydration product was the corresponding dinitrile ranging in yield from 55 to 67%. Only *n*-propylmalondinitrile was subjected to proof of structure since all of the dinitriles, with the exception of *n*-butylmalondinitrile, are reported in the literature. In each acylation attempted at a temperature between room and reflux temperature, a very small yield of 2,4-dinitrophenylhydrazone derivative was obtained from the crude ammonium chloride. This result was interpreted as an indication of a ketonic compound, and hence of very limited carbon acylation.

At reflux temperatures, malonamide, ethylmalonamide, and propylmalonamide failed to give any acylated product, and since no starting material was recovered, it was presumed that each amide was converted to its corresponding dinitrile. Varying the molar ratio of the amide to the acylating agent did not change the results. However, at reflux temperature, in the presence of acetyl chloride and pyridine, *n*-butylmalonamide underwent N-acylation to give N-acetyl-α-cyanocaproamide (I); the structure of (I) was proved by means of elemental and infrared analysis and degradation to caproamide.



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The Synthesis and Pharmacology of Alkyl Esters of N-Substituted Aminoacylaminobenzoic Acids II*

By ELIAS EPSTEIN and DANIEL KAMINSKY

Alkyl esters of N-substituted aminoacetyl-, aminopropionyl-, γ-aminobutyryl-, and δ-aminovalerylaminobenzoic acids were prepared. They, as well as previously described compounds in this series, were tested as salts for anesthetic potency, toxicity, and for irritation on experimental animals. Several of these compounds had a sufficiently high relative anesthetic efficiency (ratio of relative potency to relative toxicity) when compared to procaine hydrochloride to warrant further investigation.

IN OUR PREVIOUS PAPER (1) we described several alkyl esters of *o*- and *p*-N-substituted aminoacetyl- and aminopropionylaminobenzoic acids which showed sufficient promise as local anesthetics to warrant clinical trials. Gupta, *et al.* (2), have also prepared several members of this group of compounds. In our continued search for more effective local anesthetics, additional compounds of this type were prepared. The series has been extended to include the *m*-N-substituted aminoacetyl- and the *p*-N-substituted γ-aminobutyryl- and δ-aminovalerylaminobenzoic acid esters. These compounds, as well as those previously described (1), were tested for potency, toxicity, and irritation on laboratory animals.

Table I lists the boiling or melting points and refractive indexes of the nitroesters together with the boiling or melting points, refractive indexes, densities, molecular refractions, and analyses of the corresponding amino esters. Table II lists the chloracylaminobenzoates with their melting points and analyses, while Table III lists the melting points, analyses, and molecular weight determinations of the hydrochlorides of the alkyl esters of the N-substituted aminoacylaminobenzoic acids.

EXPERIMENTAL

The method of preparation consisted of treating a chloroacyl chloride with an ester of aminobenzoic acid and subsequent condensation of the resulting chloranilide with a primary or secondary amine to yield the anesthetic compound, as described in our previous paper (1). The aminobenzoic acid esters not commercially available were prepared by treating the *p*- and *m*-nitrobenzoylchloride with the appropriate alcohol to form the nitroester, which was then reduced catalytically or with iron filings. In the instance of the tertiary amyl ester, the presence of a proton acceptor such as dimethylaniline was necessary to drive the reaction to completion, as suggested by Abramovitch, *et al.* (3), in their preparation of tertiary butyl acetate.

The chloroacetyl, α-chloropropionyl and β-chloropropionyl chlorides were obtained from commercial sources. The γ-chlorobutyryl and δ-chlorovaleryl chlorides were prepared from their respective lactones. Poor yields were obtained by treating these lactones with excess thionyl chloride at elevated temperatures. Considerably better results were obtained using a zinc chloride catalyst, as suggested by a German patent (4).

Low yields were obtained for the chlorobutyryl and chlorovaleryl anilines by the method used and previously described (1) for the preparation of the chloroacetyl- and chloropropionylanilines. The use of a proton acceptor, such as dimethylaniline with a modified procedure, gave excellent yields.

***t*-Amyl *p*-Nitrobenzoate.**—A solution of 205 Gm. (1.1 mole) of *p*-nitrobenzoyl chloride in 700 ml. of dry benzene was added, dropwise, over a period of one hour, to a stirred solution of 88 Gm. (1.0 mole) of *t*-amyl alcohol in 121 Gm. (1.0 mole) of freshly distilled dimethylaniline. The mixture was stirred for an additional hour and then refluxed for two hours. After cooling, the mixture was successively washed with three 100-ml. portions of 5% hydrochloric acid, two 100-ml. portions of 10% sodium hydroxide, and two 100-ml. portions of water. The benzene was removed by distillation and the residue distilled; b. p. 90–92° (25μ) yield 187 Gm. (79%). Recrystallization from isopropyl alcohol yielded light yellow crystals, m. p. 54–56°.

γ-Chlorobutyryl Chloride.—A solution of 129 Gm. (1.5 mole) γ-butyrolactone and 5 Gm. anhydrous zinc chloride in 197 Gm. (1.65 mole) thionyl chloride was maintained at 50–60° for two hours. After twelve hours at room temperature the solution was distilled; b. p. 73–74° (15 mm.), lit. (4) 76–77° (18 mm.); yield 117 Gm. (55%) as a colorless oil.

Butyl Ester of *p*-(γ-Chlorobutyrylamino)-benzoic Acid.—To a stirred solution of 55 Gm. (0.3 mole) of butyl *p*-aminobenzoate in 500 ml. ether was added 50 Gm. (0.35 mole) of γ-chlorobutyryl chloride. After stirring the resulting thick slurry for one hour, 48 Gm. (0.4 mole) of N,N-dimethylaniline in 200 ml. ether was added, dropwise, keeping the temperature below 38° with continued stirring over a period of one-half hour. The mixture was stirred for an additional hour and then extracted with four 250-ml. portions of 10% hydrochloric acid and once with water. Evaporation of the ether layer yielded 79 Gm. of a light yellow product. Recrystallization from benzene yielded 70 Gm. (79%), m. p. 91–92°, as white crystals.

* Received July 19, 1958, from the Research Laboratories of Novocol Chemical Mfg. Co., Inc., Brooklyn, N. Y.

The authors are indebted to Richard Sriubas for assistance in the analyses and to Ann Hartman for assistance in the pharmacological testing of these compounds.

TABLE I.—ALKYL NITRO- AND AMINOBENZOATES[a]

Alkyl	Position	Nitro Esters b. p., ° C., (m. p.)	Nitro Esters n_D^{20}	Amino Esters b. p., ° C., (m. p.)	Amino Esters n_D^{20}	Amino Esters d_{20}^{20}	MR, Theory	MR, Found	Formula	Nitrogen, % Theory	Nitrogen, % Found
C_2H_5	*m*	(43–44)[b]	...	84–85/20μ[c]	1.5499	1.173	46.12	45.33	$C_{14}H_{19}O_2N$	8.48	8.31
i-C_3H_7	*m*	77–78/20μ	1.5210	97–99/20μ	1.5436	1.060	50.74	51.95	$C_{16}H_{23}O_2N$	7.83	7.78
C_4H_9	*m*	90–92/25μ	1.5215	95–96/20μ[d]	1.5424	1.074	55.36	56.80	$C_{17}H_{25}O_2N$	7.25	7.09
i-C_4H_9	*m*	80–87/30μ	1.5179	93–94/25μ	1.5399	1.065	55.36	56.68	$C_{17}H_{25}O_2N$	7.25	7.15
C_5H_{11}	*m*	101–102/30μ	1.5171	106–107/25μ	1.5362	1.054	59.98	61.21	$C_{18}H_{27}O_2N$	6.76	6.86
s-C_5H_{11}	*m*	93–95/20μ	1.5162	97–98/30μ	1.5322	1.042	59.98	61.65	$C_{18}H_{27}O_2N$	6.76	6.63
t-C_5H_{11}	*p*	90–92/25μ (54–56)	...	(89–91)	...	...	...	...	$C_{18}H_{27}O_2N$	6.76	6.60
C_6H_{13}	*p*	108–110/20μ[e]	1.5156	(62–64)	...	...	...	...	$C_{19}H_{29}O_2N$	6.33	6.18

[a] Amino esters used as intermediates and not recorded here are commercially available or have been previously prepared by us (1).
[b] Reported (5) 43°.
[c] Reported (6) b. p. 204°.
[d] Reported (7) b. p. 215°.
[e] Reported (8) b. p. 175–177° at 8 mm n_D^{20} 1.5153.

PHARMACOLOGY

A pharmacological screening of these compounds for use as local anesthetics was conducted and the results of the more promising compounds noted in Table IV. The hydrochloride salt of about one-third of these compounds was too insoluble and the gluconate salt was substituted. Since the anesthetic potency and toxicity are primarily functions of the concentration of the anesthetic base, the gluconate salt was evaluated on the basis of the equivalent amount of the hydrochloride.

Evidences of irritation were checked after topical application to the eye and on intradermal injection of the rabbit. The toxicity was determined on white mice, using both the intraperitoneal and subcutaneous mode of injection. Potency was determined by: blocking the sciatic nerve of the intact guinea pig for conduction anesthesia, application to the rabbit eye for topical anesthesia, and with the wheal test on the guinea pig for infiltration anesthesia. A detailed description of these tests has been previously published (9).

To compare the value of these compounds as local anesthetics with each other and with procaine hydrochloride, we used the quotient of the relative potency as determined by the guinea pig wheal test to the relative toxicity as determined by the intraperitoneal injection on white mice. The guinea pig wheal test for anesthetic potency gives a close correlation to clinical findings with commercially used anesthetics (10). The intraperitoneal toxicity on white mice approximates a mean value between the intravenous toxicity, where absorption of the drug is rapid, and the subcutaneous toxicity, where absorption is delayed.

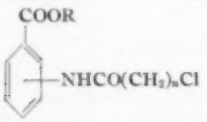
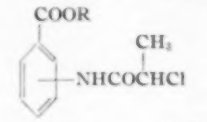
Since we were screening a rather large number of compounds for their potential value as local anesthetics, a minimum number of animals was used, and the values listed in Table IV should be considered as only semiquantitative. For the determination of potency in the rabbit eye, an average of four eyes was used for each compound. An average of eight wheals and six legs was used for the wheal and sciatic nerve tests, respectively, on the guinea pig. The subcutaneous and intraperitoneal toxicities were conducted at three critical dose levels, using three animals at each level. The critical dose is one near or at the LD_{50}. In describing irritation, 0 stands for little or no irritation, + for moderate irritation, ++ severe irritation. The detailed description of these levels of irritation is noted in the literature (9, 11).

DISCUSSION AND SUMMARY

Sixty alkyl esters of N-substituted aminoacylaminobenzoic acids were prepared. They, together with sixty-nine similar compounds previously reported, were tested pharmacologically for potential value as local anesthetics.

Although a few general rules have been postulated correlating physiological activity of benzoic acid esters with their molecular structure (12), little light has been shed on this relationship with anilides. In either case, it has

TABLE II.—CHLOROACYLAMINOBENZOATES[a]

R	Position	n	M. p., °C.	Formula	Chlorine, % Calcd.	Chlorine, % Found
						
C_2H_5	*m*	1	81–82	$C_{11}H_{12}O_3NCl$	14.67	14.49
C_2H_5	*p*	3	98–99	$C_{13}H_{16}O_3NCl$	13.15	13.05
i-C_3H_7	*m*	1	98–100	$C_{12}H_{14}O_3NCl$	13.87	13.77
C_4H_9	*m*	1	68–70	$C_{13}H_{16}O_3NCl$	13.15	12.99
C_4H_9	*p*	3	91–92	$C_{15}H_{20}O_3NCl$	11.91	11.78
C_4H_9	*p*	4	63–64	$C_{16}H_{22}O_3NCl$	11.37	11.38
i-C_4H_9	*m*	1	96–97	$C_{13}H_{16}O_3NCl$	13.15	13.18
i-C_4H_9	*p*	3	135–136	$C_{15}H_{20}O_3NCl$	11.91	11.79
C_5H_{11}	*m*	1	65–67[b]	$C_{14}H_{18}O_3NCl$	12.50	12.41
C_5H_{11}	*p*	2	87–88	$C_{14}H_{18}O_3NCl$	11.91	11.96
s-C_5H_{11}	*m*	1	[c]	$C_{14}H_{18}O_3NCl$	12.50	12.57
t-C_5H_{11}	*p*	1	114–116	$C_{14}H_{18}O_3NCl$	12.50	12.38
C_6H_{13}	*p*	1	81–83	$C_{15}H_{20}O_3NCl$	11.91	11.79
						
C_2H_5	*o*		44–45[d]	$C_{12}H_{14}O_3NCl$	13.87	13.89
C_4H_9	*p*		75–77	$C_{14}H_{18}O_3NCl$	12.50	12.38

[a] The chloroacylaminobenzoates used as intermediates and not recorded here were previously prepared by us (1). [b] B. p. 166–168°/60μ. [c] B. p. 165–167°/25μ, n_D^{25} 1.5429. [d] B. p. 115–116°/60μ.

been difficult to predict the usefulness of a compound as a local anesthetic from its structure. On screening these alkyl aminoacylaminobenzoates, we found that this uncertainty persisted.

The relative local anesthetic efficiency, the quotient of the relative potency to the relative toxicity, can be affected by many unrelated phenomena. Rates of absorption and detoxification, solubility at the pH of the body, vasomotory effect on the capillaries, and other obscure effects play an important role. We noted that some of our compounds precipitated at body pH and in many cases this appeared to be a direct cause of irritation, lower toxicity, and longer duration of the anesthetic effect. Since precipitation occurred more readily at higher concentrations, the guinea pig wheal test had an advantage over the sciatic nerve test, as the concentrations used were only one-fifth as great.

For the purpose of correlating molecular structure to physiological activity, we used the relative intraperitoneal toxicity in white mice, the relative potency as determined by the guinea pig wheal test, and the irritation as observed on both the rabbit eye and skin. The following generalizations can be made:

1. Effect of varying the ester group (R). (*a*) Potency and irritation increased with increasing molecular weight, with the straight chains being more potent and irritating than the branched chains. The methyl ester compounds were exceptions, being more potent than the ethyl esters. (*b*) Molecular size or branching of the ester group had little effect on toxicity. (*c*) Efficiency increased with increasing molecular weight and reached a maximum at five carbon atoms.

2. Effect of varying the relative position of the ester with the anilide group (*ortho*, *meta*, *para*). Although the *meta* derivatives of the aminobenzoic acid esters have been reported (13) to be much less toxic and more efficient than the corresponding *para* derivatives, this group of compounds had a reverse relationship. (*a*) With respect to potency and irritation, the *meta*> *ortho*> *para*. (*b*) With respect to toxicity and anesthetic efficiency, the *ortho*> *meta*> *para*.

3. Effect of varying the acyl chain. (*a*) Branched chain acyl compounds were slightly less toxic and irritating, equal in potency and greater in efficiency than the straight chain acyl compounds of the same number of carbon atoms in the chain. (*b*) Increasing the length of the acyl chain (*n*) increased the irritation and decreased the anesthetic efficiency.

We were not able to obtain a clearcut relationship of *n* with respect to potency and toxicity.

4. Effect of varying the terminal alkylamino

TABLE III.—ALKYL ESTERS OF N-SUBSTITUTED AMINOACYLAMINOBENZOIC ACID HYDROCHLORIDES

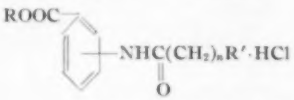
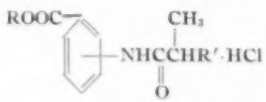
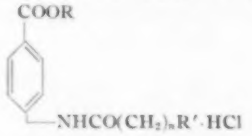
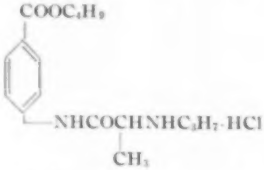
R	R′	n	Position	M. p., °C.	Formula	Chlorine, % Calcd.	Chlorine, % Found	Mol. wt. Calcd.	Mol. wt. Found
									
C_2H_5	NHC_2H_5	1	*o*	205–207	$C_{13}H_{19}O_3N_2Cl$	12.36	12.22	287	281
C_2H_5	NHC_3H_5	2	*o*	167–168	$C_{14}H_{21}O_3N_2Cl$	11.79	11.70	301	297
C_2H_5	NHC_3H_7	1	*o*	174–176	$C_{14}H_{21}O_3N_2Cl$	11.79	11.82	301	301
C_2H_5	NHC_3H_7	2	*o*	169–171	$C_{15}H_{23}O_3N_2Cl$	11.26	11.15	315	321
C_2H_5	NHC_3H_7(iso)	1	*o*	172–175	$C_{14}H_{21}O_3N_2Cl$	11.79	11.75	301	299
C_2H_5	NHC_3H_7(iso)	2	*o*	185–186	$C_{15}H_{23}O_3N_2Cl$	11.26	11.31	315	322
C_2H_5	NHC_4H_9(iso)	1	*m*	211–213	$C_{15}H_{23}O_3N_2Cl$	11.26	11.20	315	314
C_2H_5	NHC_4H_9(iso)	3	*p*	174–176	$C_{17}H_{27}O_3N_2Cl$	10.34	10.48	343	...
C_2H_5	$N(C_2H_5)_2$	1	*m*	122–124	$C_{15}H_{23}O_3N_2Cl$	11.26	11.22	315	313
C_2H_5	$N(C_2H_5)_2$	3	*p*	152–154	$C_{17}H_{27}O_3N_2Cl$	10.34	10.16	343	...
C_2H_5	Morpholino	1	*m*	199–201	$C_{15}H_{21}O_4N_2Cl$	10.75	10.89	329	327
i-C_3H_7	NHC_2H_5	1	*m*	208–209	$C_{14}H_{21}O_3N_2Cl$	11.79	11.75	301	309
i-C_3H_7	NHC_4H_9	1	*m*	198–200	$C_{16}H_{25}O_3N_2Cl$	10.78	10.74	329	329
i-C_3H_7	NHC_4H_9(iso)	1	*m*	218–220	$C_{16}H_{25}O_3N_2Cl$	10.78	11.02	329	331
i-C_3H_7	NHC_4H_9(tert)	1	*m*	250–252	$C_{16}H_{25}O_3N_2Cl$	10.78	10.72	329	325
i-C_3H_7	$N(C_2H_5)_2$	1	*m*	147–149	$C_{16}H_{25}O_3N_2Cl$	10.78	10.91	329	321
i-C_3H_7	Morpholino	1	*m*	200–201	$C_{16}H_{23}O_4N_2Cl$	10 34	10.40	343	344
C_4H_9	NHC_4H_9(iso)	1	*m*	168–169	$C_{17}H_{27}O_3N_2Cl$	10.34	10.47	343	337
C_4H_9	NHC_4H_9(iso)	3	*p*	184–185	$C_{19}H_{31}O_3N_2Cl$	9.56	9.48	371	379
C_4H_9	NHC_4H_9(iso)	4	*p*	198–199	$C_{20}H_{33}O_3N_2Cl$	9.21	9.15	385	394
C_4H_9	$N(C_2H_5)_2$	1	*m*	99–102	$C_{17}H_{27}O_3N_2Cl$	10.34	10.18	343	334
C_4H_9	$N(C_2H_5)_2$	3	*p*	155–156	$C_{19}H_{31}O_3N_2Cl$	9.56	9.48	371	...
C_4H_9	$N(C_2H_5)_2$	4	*p*	122–126	$C_{20}H_{33}O_3N_2Cl$	9.21	9.34	385	...
C_4H_9	Morpholino	1	*m*	191–194	$C_{17}H_{25}O_4N_2Cl$	9.93	9.89	357	354
i-C_4H_9	NHC_2H_5	1	*m*	129–134	$C_{15}H_{23}O_3N_2Cl$	11.26	11.45	315	308
i-C_4H_9	NHC_3H_5	2	*p*	175–176	$C_{16}H_{25}O_3N_2Cl$	10.78	10.70	329	330
i-C_4H_9	NHC_3H_7	1	*m*	196–198	$C_{16}H_{25}O_3N_2Cl$	10.78	10.63	329	324
i-C_4H_9	NHC_4H_9(iso)	1	*m*	201–203	$C_{17}H_{27}O_3N_2Cl$	10.34	10.27	343	338
i-C_4H_9	NHC_4H_9(iso)	3	*p*	187–188	$C_{19}H_{31}O_3N_2Cl$	9.56	9.42	371	382
i-C_4H_9	$N(C_2H_5)_2$	1	*m*	134–136	$C_{17}H_{27}O_3N_2Cl$	10.34	10.31	343	339
i-C_4H_9	Piperidino	1	*m*	152–154	$C_{18}H_{27}O_3N_2Cl$	10.00	9.89	355	357
C_5H_{11}	$NHCH_3$	1	*p*	171–173	$C_{15}H_{23}O_3N_2Cl$	11.26	11.12	315	309
C_5H_{11}	NHC_2H_5	1	*m*	166–168	$C_{16}H_{25}O_3N_2Cl$	10.78	10.99	329	322
C_5H_{11}	NHC_3H_5	2	*p*	194–196	$C_{17}H_{27}O_3N_2Cl$	10.34	10.18	343	343
C_5H_{11}	NHC_3H_7	1	*m*	169–170	$C_{17}H_{27}O_3N_2Cl$	10.34	10.23	343	339
C_5H_{11}	NHC_3H_7	1	*p*	212–214	$C_{17}H_{27}O_3N_2Cl$	10.34	10.30	343	343
C_5H_{11}	NHC_4H_9	1	*m*	169–172	$C_{18}H_{29}O_3N_2Cl$	9.93	9.84	357	355
C_5H_{11}	NHC_4H_9(iso)	1	*m*	162–164	$C_{18}H_{29}O_3N_2Cl$	9 93	10.02	357	352
C_5H_{11}	NHC_4H_9(iso)	2	*p*	181–183	$C_{19}H_{31}O_3N_2Cl$	9.56	9.62	371	369
C_5H_{11}	NHC_4H_9(tert)	1	*p*	245–247	$C_{18}H_{29}O_3N_2Cl$	9.93	9.96	357	357
C_5H_{11}	NHC_5H_{11}	1	*p*	260–263	$C_{19}H_{31}O_3N_2Cl$	9.56	9.56	371	363
C_5H_{11}	$N(C_2H_5)_2$	1	*m*	107–109	$C_{18}H_{29}O_3N_2Cl$	9.93	9.88	357	355
C_5H_{11}	$N(C_2H_5)_2$	2	*p*	124–127	$C_{19}H_{31}O_3N_2Cl$	9.56	9.63	371	368
C_5H_{11}	Morpholino	1	*m*	186–189	$C_{18}H_{27}O_4N_2Cl$	9.56	9.66	371	375
s-C_5H_{11}	NHC_3H_7	1	*m*	172–174	$C_{17}H_{27}O_3N_2Cl$	10.34	10.42	343	342
s-C_5H_{11}	NHC_4H_9(iso)	1	*m*	170–172	$C_{18}H_{29}O_3N_2Cl$	9.93	9.87	357	352
s-C_5H_{11}	Morpholino	1	*m*	171–174	$C_{18}H_{27}O_4N_2Cl$	9.56	9.42	371	369
t-C_5H_{11}	NHC_3H_7	1	*p*	225–228	$C_{17}H_{27}O_3N_2Cl$	10.34	10.26	343	337
t-C_5H_{11}	$N(C_2H_5)_2$	1	*p*	227–229	$C_{18}H_{29}O_3N_2Cl$	9.93	9.90	357	356
t-C_5H_{11}	Morpholino	1	*p*	182–184	$C_{18}H_{27}O_4N_2Cl$	9.56	9.43	371	362
C_6H_{13}	NHC_3H_7	1	*p*	284–286	$C_{18}H_{29}O_3N_2Cl$	9.93	9.85	357	353
C_6H_{13}	NHC_3H_7(iso)	1	*p*	242–244	$C_{18}H_{29}O_3N_2Cl$	9.93	9.90	357	351
C_6H_{13}	NHC_4H_9(iso)	1	*p*	213–215	$C_{19}H_{31}O_3N_2Cl$	9.56	9.73	371	374
C_6H_{13}	$N(C_2H_5)_2$	1	*p*	178–182	$C_{19}H_{31}O_3N_2Cl$	9.56	9.41	371	364
									
C_2H_5	NHC_4H_9(iso)		*o*	114–117	$C_{16}H_{25}O_3N_2Cl$	10.78	10.69	329	323
C_2H_5	Morpholino		*o*	190–193	$C_{14}H_{23}O_4N_2Cl$	10.34	10.24	343	338
C_3H_9	NHC_3H_7		*p*	175–177	$C_{17}H_{27}O_3N_2Cl$	10.34	10.31	343	346
C_4H_9	NHC_4H_9(iso)		*p*	160–162	$C_{18}H_{29}O_3N_2Cl$	9.93	9.99	357	359
C_4H_9	$N(C_2H_5)_2$		*p*	147–149	$C_{18}H_{29}O_3N_2Cl$	9.93	10.10	357	360
C_4H_9	Morpholino		*p*	172–174	$C_{18}H_{27}O_4N_2Cl$	9.56	9.47	371	370

TABLE IV.—PHARMACOLOGY OF ALKYL ESTERS OF N-SUBSTITUTED AMINOACYLAMINOBENZOIC ACID HYDROCHLORIDES

R	R′	n Position	Relative Potency: Rabbit eye[a]	Guinea pig[b] Wheal	Guinea pig[b] Sciatic Nerve No. Ep.	Guinea pig[b] Sciatic Nerve W. Ep.	Relative Toxicity, i. p.	Relative Toxicity, s. c.	Irritation Eye	Irritation Skin	Relative Efficiency
											
C_2H_5	NHC_3H_7(iso)	1 *o*	1.1	2.4	1	0.1	0.4	0.5	+	+	6.0
C_2H_5	NHC_4H_9	2 *o*	2.4	10	3	4	0.8	0.9	+	0	13
C_2H_5	NHC_4H_9(iso)	1 *o*	0.1	1.3	6	2	0.2	0.2	0	++	6.5
C_2H_5	$N(C_2H_5)_2$	1 *o*	0.2	1.9	3	4	0.4	0.5	+	+	4.8
C_2H_5	Cyclohexylamino	1 *p*	0.4	1.5	1	3	0.1	<0.3[c]	0	+	15
i-C_3H_7	NHC_3H_7	2 *p*	3.8	3.0	4	1	0.8	0.6	++	0	3.8
i-C_3H_7	NHC_4H_9(iso)	1 *m*	4.0	6.4	2	1	0.9	0.8	+	0	7.1
C_4H_9	NHC_4H_9	2 *p*	0.9	1.1	3	1	0.3	0.2	+	0	3.7
C_4H_9	Morpholino	2 *p*	0.9	1.3	2	1	0.2	0.3	+	0	6.5
i-C_4H_9	NHC_4H_9(iso)	2 *p*	1.7	1.9	0.9	0.8	0.4	0.3	+	0	4.8
i-C_4H_9	$N(C_2H_5)_2$	1 *m*	1.0	2.5	1	0.4	0.1	<0.2	0	++	25
i-C_4H_9	$N(C_2H_5)_2$	2 *p*	0.5	2.5	3	2	0.1	<0.2	0	0	25
i-C_4H_9	Morpholino	1 *p*	0.8	1.1	2	0.5	0.1	0.1	+	0	11
i-C_4H_9	Morpholino	2 *p*	1.0	1.0	3	2	0.1	<0.2	+	0	10
i-C_4H_9	Cyclohexyl-methylamino	1 *p*	1.6	1.9	5	1	0.3	0.3	0	+	6.3
i-C_4H_9	Benzylamino	1 *p*	0.3	1.9	4	1	0.1	0.2	0	+	19
C_5H_{11}	NHC_2H_5	1 *m*	1.2	7.6	2	0.5	1.5	1.9	+	0	5.1
C_5H_{11}	NHC_2H_5	1 *p*	1.2	5.1	2	4	0.4	<0.3	+	0	13
C_5H_{11}	NHC_3H_7	1 *p*	1.8	1.5	2	1	0.3	0.3	+	+	5.0
C_5H_{11}	NHC_4H_9(iso)	1 *m*	1.0	4.8	1	1	0.6	0.3	+	+	8.0
C_5H_{11}	NHC_4H_9(tert)	1 *p*	2.5	1.0	0.9	0.7	0.2	0.2	+	0	5.0
C_5H_{11}	$N(C_2H_5)_2$	1 *p*	0.9	1.3	2	2	0.1	0.2	+	+	13
											
			1.0	1.5	1	1	0.4	0.5	0	+	3.8

[a] Cocaine = 1. [b] Procaine = 1. [c] Values of < no. used when solubility was too low to obtain the final value.

group (R′). There was little apparent correlation between the structure of this group and physiological activity other than the tertiary alkylamino groups being less toxic than the corresponding secondary group with the same number of carbon atoms.

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Kinetics of Formation of Anhydrovitamin A From Vitamin A Alcohol and Its Acetate*

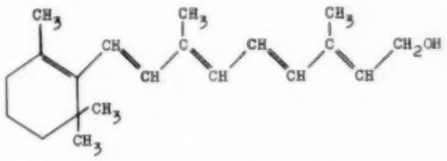
By TAKERU HIGUCHI and JEROME A. REINSTEIN†

Of the several pathways by which vitamin A and its derivatives can degrade, the present study is concerned with those leading to the formation of anhydrovitamin A. Results indicate (*a*) anhydro formation does not occur readily with vitamin A alcohol in the absence of a strong catalyst such as hydrogen chloride, (*b*) the reaction proceeds at a significant rate for solutions of the acetate in both alcoholic and hydroalcoholic systems, (*c*) the conversion of the acetate to the anhydro form is much more rapid in the presence of water than in its absence, and (*d*) formation from the acetate does not occur in ether or hydrocarbon solvent in the absence of catalytic agents. Catalytic behavior of hydrogen chloride, perchloric acid, and acetic acid were also studied. The results on experiments made with pyridine and sodium hydroxide as possible inhibitors are also presented.

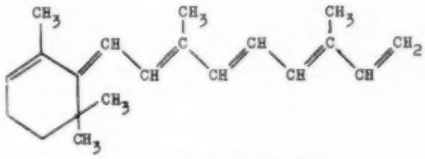
ALTHOUGH from a pharmaceutical and economic standpoint reactions responsible for loss in activity of vitamin A and its derivatives are of considerable importance, relatively little basic information is available concerning the mechanism of breakdown of this vitamin under practical conditions. The facts that we do have, however, point to several broad general class reactions which may be responsible for the observed instability of the polyenoid vitamin. These are (*a*) free radical mediated oxidation and polymerization reactions, (*b*) photochemical reactions, (*c*) isomerization, (*d*) addition reactions involving the vitamin's double bonds and protonated solvents, and (*e*) elimination reactions resulting in the formation of anhydrovitamin A. The present communication is concerned with the results of an investigation into some aspects of the fifth route of breakdown.

Since vitamin A is oftentimes formulated in vehicles containing alcoholic solvents, this study has been directed largely toward evaluation of several factors which may govern the relative rate of the elimination reaction in various hydroxylic and nonhydroxylic solvents. Some earlier work on the hydrogen chloride catalyzed reaction has been repeated and expanded.

The tendency of vitamin A and its derivatives to form anhydrovitamin A (Fig. 1) can be readily followed by ultraviolet spectrophotometry. The increase in length of the conjugated chain results in the appearance of a series of absorption peaks in the near ultraviolet in a region partly transparent for the parent compound. Fortunately



All-trans Vitamin A_1



Anhydrovitamin A

Figure 1.

the absorption spectrum of pure anhydrovitamin A has already been determined by Shantz and colleagues (1). Thus by following the increase in the absorbance of the degrading system at or near 392 mμ, the long wavelength absorption peak for the anhydro form, the kinetics of the reaction can be ascertained easily. In the present studies the long wavelength peak appeared at 386.5 mμ (Fig. 2). Apparently the displacement of the peak was caused by the absorbance of the other products present. The peak has previously been found to appear at 388 mμ (2) and 389 mμ (3).

PAST WORK

Anhydrovitamin A was first reported as a product of the action of anhydrous hydrogen chloride on vitamin A in ethanol by Edisbury, *et al* (4). They noted that this treatment resulted in the appearance of narrow bands in the ultraviolet absorption spectrum. For many years the nature of the transformation was misinterpreted and the compound was known as "cyclized vitamin A" (5). In 1943 Shantz, Cawley,

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From a thesis submitted by Jerome A. Reinstein to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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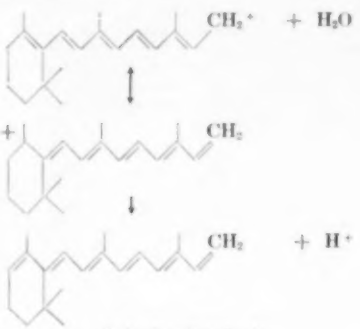
and Embree (1), and Meunier, Dulou, and Vinet (6) presented evidence that the substance was formed not by cyclization but by loss of a molecule of water from vitamin A alcohol. The compound was renamed anhydrovitamin A. It had 0.4% of the biological activity of vitamin A.

Meunier, *et al.* (6), suggested a mechanism for proton catalyzed formation of anhydrovitamin A which seems quite plausible:

Vit. A alcohol + H^+ →



Anhydrovitamin A

A compound which appeared to be an addition product of vitamin A and hydrogen chloride was obtained by Guerillot-Vinet, Meunier, Jouanneteau, and Gourevitch (7). They confirmed the observation of Shantz and colleagues (1) that prolonged action of hydrogen chloride transformed anhydrovitamin A into "isoanhydrovitamin A" and found that the action of hydrogen chloride did not stop there but caused new spectral changes in which the three bands of "isoanhydrovitamin A" were replaced by a single band at 325 mμ nearly superimposable on that of vitamin A itself. The compound, which they called "substance X," was biologically inactive.

In 1954 Oroshnik (8) proposed that "isoanhydrovitamin A" was in fact not an isomer of anhydrovitamin A, but an ethoxy addition product formed by solvation of the terminal vinyl group of anhydrovitamin A by ethanol.

The conversion of vitamin A to anhydrovitamin A has been studied as a means of determining the vitamin in natural products (9). Robeson and Baxter (10) in the course of their study of the properties of neovitamin A, compared the rates of dehydration of vitamin A and its neo isomer and found that the neo form dehydrated more slowly.

OBSERVATIONS

The elimination reaction responsible for formation of anhydrovitamin A appears to be rather sensitive to experimental conditions. The free vitamin alcohol, for example, has been shown in a previous study (11) to be highly resistant toward this reaction. Despite the considerably greater susceptibility of the alcohol toward oxidative and other free radical mediated losses, it exhibits such great resistance toward dehydration in the absence of catalysts that the reaction could only be studied conveniently in their presence.

In the present report results are given in the first part on the essentially uncatalyzed elimination reaction as occurring in several solvent systems. Since only the ester undergoes this reaction readily, the investigation was confined to this form. In the second part, the elimination behavior of both the ester and the alcohol in the presence of catalytic agents and additives is discussed.

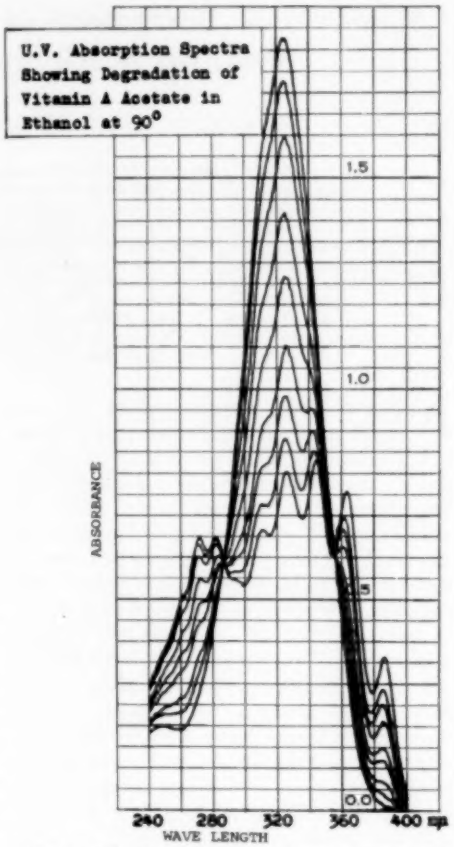


Fig. 2.—Vitamin A peak at 326 mμ decreases with time. Anhydrovitamin A peaks at 350, 367, and 387 mμ increase with time. Vitamin A epoxide peaks between 270 and 285 mμ increase with time.

Anhydrovitamin A Formation From Vitamin A Acetate in Several Solvents

Order of the Reaction with Respect to Substrate.—Though any simple mechanism leading to the formation of anhydrovitamin A by an elimination reaction would yield a first order dependency on the concentration of the initial compound, results such as shown in Fig. 3 indicated that the overall process is quite complex and may not necessarily show a first order behavior. In this plot the percentage of anhydro compound formed with respect to the initial amount of vitamin A acetate is shown as a function of time in a hydroalcoholic medium at 25°. If simple first order kinetics were being obeyed, the curve would follow the usual mathematical form

$$\text{per cent conversion} = 100\,(1 - e^{-kt}\,).$$

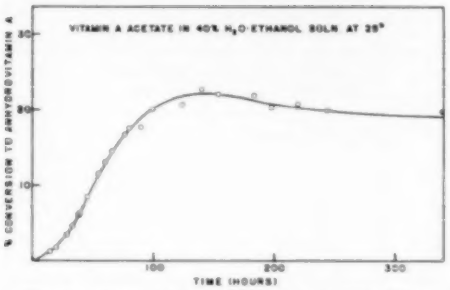


Fig. 3.—Conversion of vitamin A acetate (60 mg. per liter) in 40% ethanol: water to anhydrovitamin A at 25°.

The second derivative would always be negative. This obviously is not the case.

The exact rate dependency on the substrate concentration can be determined more directly. A series of runs were made on the acetate at varying initial concentrations as shown in Fig. 4. The initial rates of formation of the anhydro species, as calculated from the curves, were plotted against the initial concentrations of acetate. The resulting plot is shown in Fig. 5. The straight line passing through the origin indicates that the reaction is first order with respect to the substrate as expected. The particular runs were made in 40% water in ethanol, but similar results were also found in pure ethanol as shown in Fig. 6.

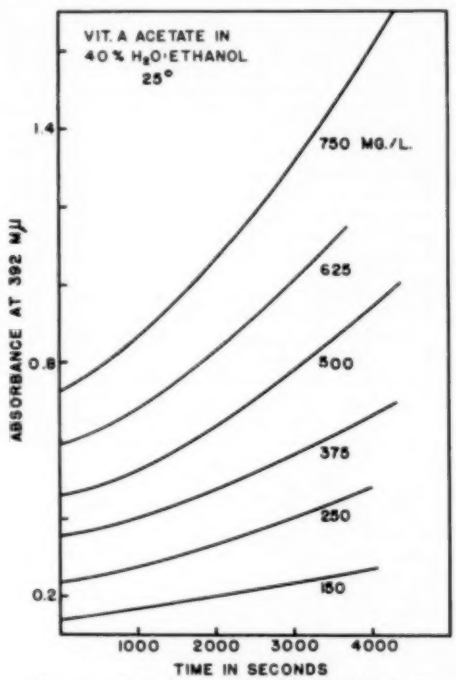


Fig. 4.—Formation of anhydrovitamin A from varying concentrations of vitamin A acetate in 40% water: ethanol at 25°. Rate of formation of peak at 392 mμ.

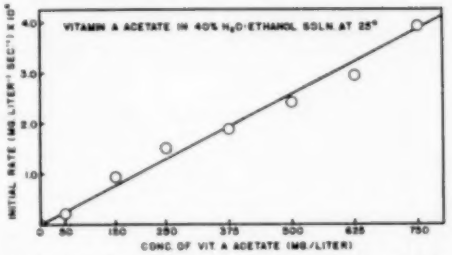


Fig. 5.—Dependence of initial rate of formation of anhydrovitamin A on concentration of vitamin A acetate in 40% water: ethanol at 25°.

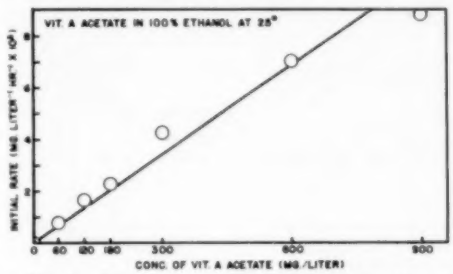


Fig. 6.—Dependence of initial rate of formation of anhydrovitamin A on concentration of vitamin A acetate in pure ethanol at 25°.

Dependence of the Rate of Formation of Anhydrovitamin A on the Solvent Composition.—The rate of formation of the anhydro form from the ester appears to be greatly dependent on the nature of the solvent used in carrying out the reaction. In the absence of a hydroxylic solvent the rate is reduced to negligible proportions. Studies in heptane and isopropyl ether made in connection with this investigation indicated no trace of the reaction, all breakdown apparently occurring through oxidation or related routes.

That anhydrovitamin A could be obtained from vitamin A ester in ethanol was noted previously by Gray and Cawley (11) in 1942. Based on their observations Shantz and colleagues (1) found that when "vitamin A ester concentrate is refluxed. . .with. . .a lower alkyl alcohol . . . the vitamin A ester breaks down into anhydrovitamin A, free fatty acid, and other products." At 25° in 100% ethanol the present study showed that the rate of conversion of vitamin A acetate to the anhydro form was approximately 1.6×10^{-3}% per hour (Fig. 7).

For reactions run in hydroalcoholic solutions, the rate of formation of anhydrovitamin A was found to increase very rapidly as the per cent water in ethanol increased. Data for the variation in initial rate of anhydro A formation with concentration of water in ethanol are shown graphically as a log-log plot in Fig. 8. The rate in 100% ethanol was subtracted from the total rate to give the net rate due to water. The slope of the resulting curve at any water concentration, it is evident, is equal to the order of the reaction with respect to water at that concentration.

It can be seen that the effect of water is rather complex since the order is constantly changing. From much less than one at 2% water, the order increases to one at 5%, and then to two at 20%. At 30% the slope appears to incline sharply, and the or-

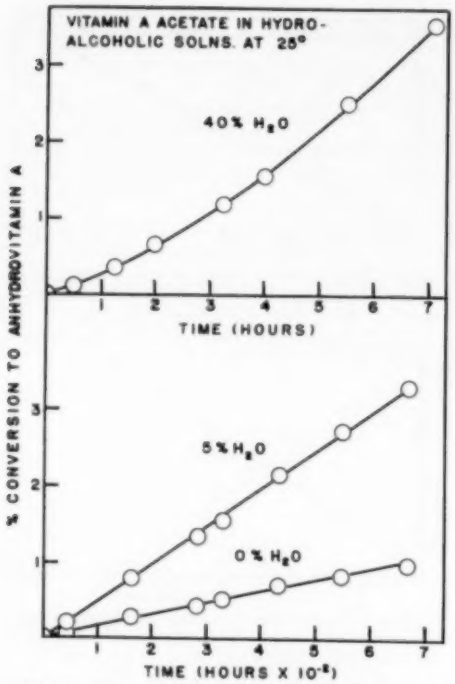


Fig. 7.—Rate of conversion of vitamin A acetate to anhydrovitamin A in ethanol with varying proportions of water at 25°.

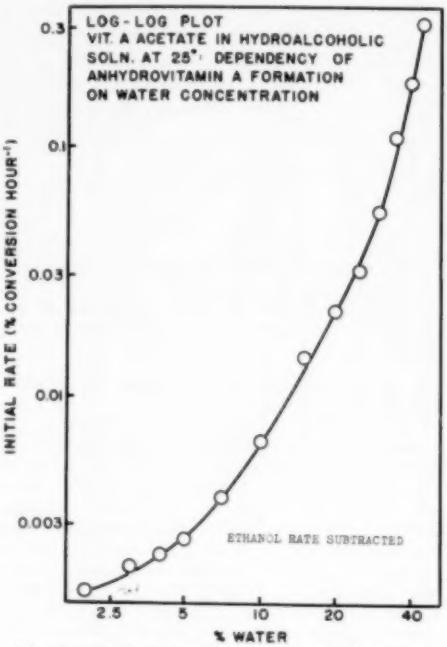

LOG-LOG PLOT
VIT. A ACETATE IN HYDROALCOHOLIC SOLN. AT 25°: DEPENDENCY OF ANHYDROVITAMIN A FORMATION ON WATER CONCENTRATION
INITIAL RATE (% CONVERSION HOUR⁻¹)
ETHANOL RATE SUBTRACTED
% WATER

Fig. 8.—Variation in initial rate of anhydrovitamin A formation with concentration of water in ethanol at 25°.

der reaches five at 45% water. This very great dependence of the order on high water concentrations is difficult to explain. One possibility is that there is a change in mechanism at the higher concentrations. The data in Fig. 7 lend some support to this. At low concentrations of water in ethanol (0% and 5% are shown), the plot of per cent conversion *vs.* time approximated a straight line, which would be expected for the first few per cent conversion. But the plot for 40% water in ethanol had a positive second derivative. Curves of this type as previously pointed out are shown by reactions which are complex.

When the time axis of the latter curve was extended to 300 hours (Fig. 3), the per cent conversion reached a peak at about 23% and then declined because of degradation of the anhydro compound. From the low yield[1] it would appear that there are other products of the degradation, probably addition compounds, which are formed concomitantly.[2]

Anhydrovitamin A Formation From Vitamin A Alcohol and Esters in the Presence of Various Additives

The effects of hydrogen chloride, perchloric acid, sodium hydroxide, and pyridine were studied because they were convenient for investigating acid and base effects which might give some insight into the general elimination reaction. The effects of acetic acid and alpha-tocopherol on the reaction were studied because substances of their type might be found in pharmaceutical formulations or appear on storage.

Effect of Hydrogen Chloride on the Formation of Anhydrovitamin A.—The qualitative effect of hydrogen chloride on the dehydration of vitamin A alcohol in ethanol was reported in the original work on anhydrovitamin A formation (1, 4). Hydrogen chloride also catalyzes the formation of the anhydro compound from vitamin A acetate, although the rate of the reaction with the alcohol is about 30 times faster than that with the ester. This relationship is rather surprising in view of the fact that in ethanol, the alcohol form of the vitamin is nearly totally resistant to the elimination reaction, whereas the ester reacts at an appreciable rate.

Catalysis by hydrogen chloride of anhydrovitamin A formation from vitamin A acetate in ethanol was studied at 25°, 30°, and 40°. The reaction was found to be first order with respect to vitamin A acetate (Table I) and with respect to hydrogen chloride (Table II). The rate expression may be stated as

$$d\,(\text{Anhydro Vit. A})/dt = k\,(\text{Vit. A Acetate})\,(\text{HCl})$$

The values obtained for the specific reaction rate, k, are given in Table III. An Arrhenius plot of logarithm of rate constant against reciprocal of the absolute temperature gave a heat of activation, ΔH, of 19.6 Kcal.

To determine whether the principal catalytic species was the proton or the hydrogen chloride molecule, the rate of formation of anhydrovitamin A from vitamin A alcohol was studied in two other systems: perchloric acid in 100% ethanol and hydrogen chloride in 40% water in ethanol.

[1] Our studies of the acid catalyzed vitamin A alcohol and acetate elimination yielded from 30 to 60% conversion. Robeson and Baxter (10) obtained 62% conversion.

[2] All the reactions responsible for degradation of vitamin A in these systems must be first order, since semilog plots indicate that the rate of overall disappearance is directly dependent on the substrate concentration.

Although perchloric acid is intrinsically a stronger acid than hydrogen chloride (12), the rate of formation of anhydro A from vitamin A in the perchloric acid catalyzed system was less than half that in the hydrogen chloride catalyzed system. This seemed to indicate that the principal catalytic species was not some protonated acidic substances but the uncharged catalyst.

The data from the second system studied pointed to the same conclusion. When the reaction was run in 40% water in ethanol, the rate of formation of the anhydro compound from vitamin A was less than one-tenth the rate in pure ethanol. In the hydroalcoholic system the hydrogen chloride would be present almost entirely as oxonium and chloride ions, whereas in the pure ethanol system there would be relatively more hydrogen chloride present as the undissociated molecule. That the rate was greater in the pure ethanol system suggested strongly that the principal catalytic species was the hydrogen chloride molecule.

TABLE I.—DEPENDENCE OF HYDROGEN CHLORIDE CATALYZED ELIMINATION ON THE CONCENTRATION OF VITAMIN A ACETATE AT 25° (0.01 *N* HCl)

Concn. of Vit. A Acetate, mg./L.	Initial Rate, mg. liter^{-1} sec.$^{-1}$
100	0.0048
200	0.0101
400	0.0200
800	0.044

TABLE II.—DEPENDENCE ON HYDROGEN CHLORIDE CONCENTRATION OF THE RATE OF ANHYDROVITAMIN A FORMATION FROM VITAMIN A ACETATE (60 MG. PER LITER)

	Initial Rate, mg. liter^{-1} sec.$^{-1}$ × 10^5	
Molar Concn. HCl	30°	40°
1×10^{-4}	5.4	14
2×10^{-4}	9.3	29
4×10^{-4}	20.0	62
6×10^{-4}	..	88
8×10^{-4}	46.0	124

TABLE III.—VITAMIN A ACETATE ELIMINATION CATALYZED BY HYDROGEN CHLORIDE

Temperature, ° C.	Specific Reaction Rate[a] liters mole^{-1} sec.$^{-1}$
25	6.2×10^{-3}
30	10.6×10^{-3}
40	30.4×10^{-3}

[a] Calculated from initial rates of formation of anhydro A

Dependence of the Rate of Hydrogen Chloride Catalyzed Anhydrovitamin A Formation on Ethanol Concentration.—It was found that the rate of hydrogen chloride catalyzed elimination in nonhydroxylic solvents was very low. In the absence of catalysts, as was stated earlier, the reaction was not detected. These studies, run at 0.01 *N* hydrogen chloride concentration for convenience, show the dependence of the hydrogen chloride catalyzed rate on the concentration of a hydroxylic solvent, ethanol. The systems studied were vitamin A alcohol in ethanol:heptane and in ethanol:isopropyl ether.

Limited solubility of hydrogen chloride in heptane prevented the use of low concentrations of ethanol in the ethanol:heptane experiments. A plot of logarithm of initial rate against logarithm of initial concentration is shown in Fig. 9. The slope of the curve is equal to the order of the reaction with respect to ethanol. In the concentration range studied (40–100% ethanol; the point at 20% ethanol is uncertain) the dehydration of vitamin A alcohol in the presence of hydrogen chloride was first order with respect to ethanol.

The dependence of the rate of dehydration on ethanol in the range 5–100% ethanol in isopropyl ether can be seen in Fig. 10. At the lower concentrations the slope of the curve indicated that the order approached two with respect to ethanol. As the concentration of ethanol was increased, the order gradually decreased until at high concentrations, the dehydration was first order with respect to ethanol.

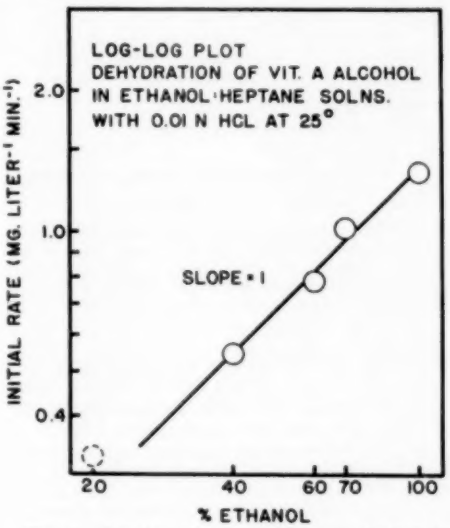


Fig. 9.—Log-log plot of vitamin A alcohol in ethanol:heptane solutions with 0.01 *N* hydrogen chloride at 25°. Shows dependence on ethanol concentration of initial rate of formation of anhydrovitamin A from vitamin A alcohol.

Effect of Pyridine on the Rate of Formation of Anhydrovitamin A.—It was thought that if the mechanism of elimination for vitamin A acetate in ethanol were proton activated, then a proton scavenger would reduce the rate. The system studied to test this possibility was vitamin A acetate (60 mg. per liter) in ethanol at 90°, alone and with 0.001 *N* pyridine. Pyridine was selected because it should tie up free protons without hydrolyzing the ester. The solutions with and without pyridine showed the same rate of formation of anhydrovitamin A. This seemed to indicate that the mechanism of formation of the anhydro compound from vitamin A acetate in ethanol was probably not exactly that suggested for proton catalyzed dehydration of vitamin A alcohol.

Effect of Sodium Hydroxide on Vitamin A Acetate in 40% Water in Ethanol.—To test the effect of strong base on the rate of anhydrovitamin A formation from vitamin A acetate, solutions were made

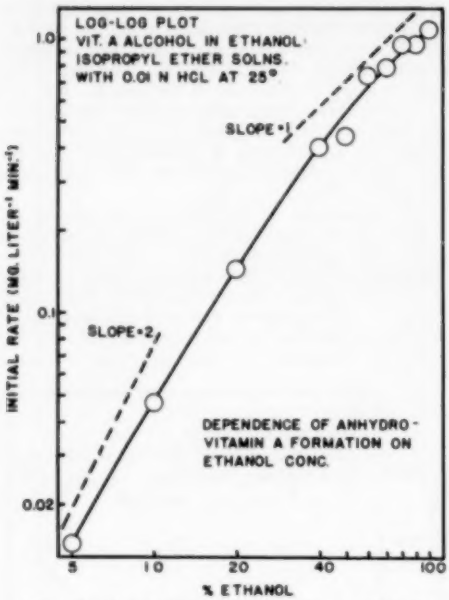


Fig. 10.—Log-log plot of vitamin A alcohol in ethanol:isopropyl ether solutions with 0.01 *N* hydrogen chloride at 25°. Shows dependence on ethanol concentration of initial rate of formation of anhydrovitamin A from vitamin A alcohol.

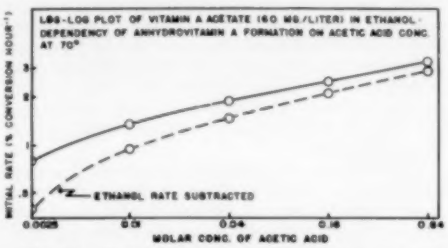


Fig. 11.—Log-log plot of the dependence on acetic acid concentration of the rate of formation of anhydrovitamin A from vitamin A acetate in ethanol.

with 60 mg. per liter of vitamin A acetate and sodium hydroxide at 0.001 *N* and 0.02 *N* concentrations. After fifty days at 25° these solutions showed very little change in their ultraviolet spectra.

If the base hydrolyzed the ester very rapidly and left vitamin A alcohol in solution, this would explain the stability toward anhydrovitamin A formation, since we have shown that the alcohol form does not degrade at that concentration in ethanol at 25° in that period of time. But in order for us not to detect any anhydro form, the hydrolysis would have had to occur within minutes, and this seemed very unlikely at room temperature. Consequently, sodium hydroxide appeared to have stabilized the acetate against elimination. This is contrary to what one might expect of base, since it usually catalyzes elimination reactions.

Dependence on Acetic Acid Concentration of the Rate of Anhydrovitamin A Formation in Ethanol.—Dependence on acetic acid concentration of the rate of formation of anhydrovitamin A from the acetate in ethanol was studied at 70° over a 265-fold range. The results are shown in Fig. 11. The solid line represents the overall rate, the dashed line the difference between the observed rate and that of alcohol alone. Over the range studied, the dependence of the rate on acetic acid appears to vary from first order at 0.0025 *N* acetic acid to one-fourth at 0.64 *N*.

The study showed that the presence of the acetic acid molecule increased the rate of conversion of vitamin A acetate to anhydrovitamin A significantly. It seems, however, that the carboxylic acid catalyzed reaction would not be responsible for the major portion of the degradation of vitamin A ester, especially in hydroalcoholic solutions.

Influence of α-Tocopherol on the Rate of Anhydrovitamin A Formation from Vitamin A Esters.—Phenolic antioxidants such as tocopherol are commonly used in vitamin A preparations to retard oxidative loss of the vitamin. There is no question that it exerts this effect in oil solutions, but there seems to be some doubt as to its effect in aqueous dispersions (13, 14).

At 0.5% concentration α-tocopherol was found to have no significant effect on the rate of formation of anhydrovitamin A from vitamin A acetate or palmitate. As one may expect the acidity of this compound was apparently too weak to endow it with any catalytic activity for the elimination reaction.

DISCUSSION

The exact nature of the mechanisms and pathways taken in the formation of anhydrovitamin A from either the acetate or the alcohol is impossible to delineate with presently available information. It is clear that the transformation is not a simple reaction and in all probability is an end result of one of many competing mechanisms. There also appears to be some evidence for formation of an intermediate species which then decomposes to form anhydrovitamin A. In all likelihood addition, isomerization, elimination, and other reactions are occurring simultaneously.

In the data collected, however, some semblance of order exists. Vitamin A alcohol in ethanol would be expected to be stable to dehydration in the absence of catalysts, as was found. If the catalysis by hydrogen chloride were by oxonium ion, the alcohol form would yield anhydrovitamin A faster than the acetate form because it could form a carbonium ion more easily. From the evidence, it appears that oxonium ion is not the principal catalytic species, but the mechanism of elimination may be similar since the alcohol form is converted to the anhydro form more readily than is the acetate in the presence of hydrogen chloride.

The acetate undergoes conversion to anhydrovitamin A in the presence of hydroxylic solvents and acids. For this compound, such solvents appear to be necessary for rapid reaction even in the presence of strong catalysts. At first glance it might seem that the elimination reaction by which vitamin A acetate is converted to the anhydro form follows a straight E_2 pathway, with the ethanol or water solvent acting as the base. If this were true, a stronger

base such as sodium hydroxide would be expected to speed up the reaction. Since it appears that sodium hydroxide is actually an inhibitor, the reaction is probably not through a simple E_2 mechanism.

PHARMACEUTICAL SIGNIFICANCE

Deterioration of vitamin A esters in pharmaceutical preparations by conversion to the anhydro form does not seem to have received much attention in the literature. Since the reaction can take place in the absence of strong catalysts in hydroxylic solvents, detection of anhydrovitamin A would appear to be a necessary part of studies on the degradation of vitamin A in solution or in dispersion in these solvents. Some studies reported up to now (13, 14) have measured only the absorption at 326 mμ, or at 620 mμ in the Carr-Price procedure, neither of which would show the presence of the anhydro form. The Carr-Price method, in particular, is not suitable for systems which form the anhydro species since the latter gives a blue color very similar in wavelength and intensity to that of vitamin A itself.

EXPERIMENTAL

Reagents.—Crystalline vitamin A alcohol, vitamin A acetate, and vitamin A palmitate (Hoffmann-La Roche); α-tocopherol (Hoffmann-La Roche); pyridine, perchloric acid, hydrochloric acid, acetic acid, sodium hydroxide—all reagent grade; *n*-heptane (Eastman, white label)—shaken with sulfuric acid, then passed through Davison silica gel (through 200 mesh); isopropyl ether (Matheson Coleman and Bell, Pract.)—peroxides removed by treatment with acidified ferrous sulfate, dried with Drierite and distilled; "absolute" alcohol, N. F.—reacted with magnesium activated by iodine and then distilled using a 10:1 reflux:distill ratio on a 50 plate Oldershaw fractionating column; water content after distillation: 0.014% (Karl Fischer titration); and double distilled water.

Apparatus.—Cary Recording Spectrophotometer Model 11MS with constant temperature jacket; constant temperature baths at 30°, 40°, 70°, and 90° ± 0.1°, and constant temperature room at 25° ± 0.5°, 10 ml. Neutraglas Color-Break Ampuls.

Procedure.—All studies were made in the absence of light. For reactions with half lives longer than about one hour, the formation of anhydrovitamin A was followed spectrophotometrically by withdrawing aliquots (for lower temperatures) or ampuls (for higher temperatures) at suitable intervals and measuring the absorbance at 392 mμ.

For reactions with half lives of the order of minutes, the appearance of the anhydro form was followed directly in a Cary spectrophotometer which had a water jacketed cell compartment to keep the temperature constant. A measured volume of substrate solution was pipetted into a silica sample cell. Then a measured volume of catalyst was pipetted into the cell, the solution shaken, placed in the sample compartment and the spectrophotometer, which was set to record at the appropriate wavelength, was switched on. The time elapsed between pipetting the catalyst into the cell and starting the recording was twelve seconds. The curve of concentration of anhydrovitamin A against time was obtained automatically.

The initial rate of formation of anhydrovitamin A was calculated from the rate of increase in absorbance at 392 mμ by the formula:

$$\text{Initial Rate of Formation of Anhydrovitamin A (mg. liter}^{-1}\text{ min.}^{-1}) = \frac{\text{Initial Increase in Absorbance per min. at 392 m}\mu}{A^{1\%}_{1\text{ cm}} \times 10^{-4}} = \frac{\text{Initial Increase in Absorbance per min. at 392 m}\mu}{3180 \times 10^{-4}}$$

CONCLUSIONS

1. The formation of anhydrovitamin A was first order with respect to vitamin A.
2. Water caused a marked increase in the rate of formation of anhydrovitamin A in hydroalcoholic solutions. The order of the reaction with respect to water varied from less than one at 2 per cent water to five at 45 per cent water.
3. Catalysis by hydrogen chloride of anhydrovitamin A formation from vitamin A acetate was first order with respect to vitamin A and with respect to hydrogen chloride. The principal catalytic species appeared to be the whole hydrogen chloride molecule rather than the hydrogen ion.
4. At concentrations above about 50 per cent ethanol, the hydrogen chloride catalyzed dehydration of vitamin A alcohol in ethanol:heptane and ethanol:isopropyl ether solutions was first order with respect to ethanol.
5. Pyridine has no effect on the rate of anhydrovitamin A formation.
6. Sodium hydroxide appeared to inhibit the formation of anhydrovitamin A in solutions of vitamin A acetate in 40 per cent water:ethanol.
7. Acetic acid caused a distinct but relatively slight increase in the rate of anhydrovitamin A formation from vitamin A acetate.
8. α-Tocopherol had no effect on the rate of anhydrovitamin A formation.

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A Chemical Study of American Tung Oil*

By YONG CHOO LEE† and W. LEWIS NOBLES

A phytochemical investigation of American tung oil which had been reported to have hypotensive properties was carried out in an attempt to isolate the active component in a pure state. 9,14-Dihydroxy-10,12-octadecadienoic acid which had been reported previously in tung oil, was isolated by using Permutit A (anion exchange resin) rather than Ionac A. A white crystalline substance, m. p. 295–297°, was obtained in low yield from the 80 per cent alcohol extract of a petroleum ether solution of tung oil. It is a substance not previously reported in tung oil and appears to be a phytosterolin (phytosterol glycoside) according to its physical and chemical properties. From the fraction not containing eleostearic acid after hydrolysis, a syrupy extract was obtained by utilizing freeze-drying; from the saponifiable fraction, eleostearic acid was isolated.

THE TUNG OIL tree is *Aleurites fordii* Hemsley, belonging to the *Euphorbiaceae*. Various steps must be taken after the nuts are harvested to obtain tung oil. These include drying in the orchards, hulling, and expression or solvent extraction of the oil. Many factors, including the degree of moisture content, type of hulling, temperature, and nature of solvent affect the quantity and quality of the oil obtained.

Tung oil plays an important role in the coating industry, but it is not used in significant amounts in the medicinal field in this country at this time. In China, it is given (1) as a remedy in insanity and in the case of metallic poisoning; also it is applied as a stimulant to carbuncles, ulcers, burns, swellings, and bruises. A refined tung oil is currently being advertised in this geographical area (southeastern United States) for promoting the healing of cuts, wounds, and abrasions.

Kaufmann (2) has reported that tung oil contains 78.5–87.1% eleostearine, 7.6–22.8% olein, and 8.5% glycerides of saturated acids. Smedley-MacLean (3) has reported that tung oil is composed entirely of C_{18} fatty acids. Recently, Hoffmann and O'Connor (4) succeeded in isolating the *alpha*- and *beta*-eleostearic acids from tung oil using ethanol as the solvent for recrystallization.

Henry and Auld (5) suggested that there may be a cyanogenetic glycoside in the fruit of tung tree and that the poisonous nature of the press cake may be due to hydrogen cyanide produced by the hydrolysis of the glycoside during purification.

Emmel (6) determined the presence of two toxic principles in the tung nut. He indicated that the first toxic principle is probably a saponin and is in the foliage, sap, and fruit. He found that the second toxic principle in tung meal is partially destroyed by heat or hydrolysis and is present in greater quantity in fresh tung meals than that in the old meals.

Carratolá (7) stated that the liquid obtained by the incision of the fruit exerts a local irritation on the skin. He found that the liquid contains a substance like a toxalbumin, a natural albuminoid which acts similarly to strychnine in the human body. He did not isolate any alkaloids from this liquid. A solution of the substance, which was heated to 90° and injected into guinea pigs, elicited no toxic response.

Bilger and Westgate (8) reported the presence of sterols in tung oil and compared them with sterols obtained from other tropical oils. They found that tung oil possessed the following properties: unsaponifiable material, 0.59%; fraction precipitated by digitonin from an alcoholic extract, 37.30%; sterol in oil, 0.22%; molecular weight of the tung sterol, 365.

In the present study, we were particularly interested in the hypotensive factor reported in tung oil by Grollman (9). He suggested that the tung oil is apparently the only oil of vegetable origin which contains a factor of this type. Also, Karnovsky (10) reported that the only vegetable fat containing any appreciable quantity of alpha-glyceryl ethers is tung oil. However, whether or not there is any relationship between these two isolated bits of information remains yet to be determined.

Davis, Conroy, and Shakespeare (11) isolated a crystalline substance in low yield from tung oil and it was shown to be 9,14-dihydroxy-10,12-octadecadienoic acid, $CH_3(CH_2)_5CHOH—CH=CH—CH=CH—CHOH—(CH_2)_7COOH$, suggested as being an artifact derived from eleostearic

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acid. The hypotensive activity of this acid, reported by Grollman (9), was not confirmed by these workers.

This study was designed to attempt the separation, isolation, and identification of certain of the chemical constituents of tung oil. Particular emphasis was placed on those constituents possessing potential medical value. Specifically, attempts were made to isolate the hypotensive factor reported by Grollman (9) in larger quantities than ever before, identify it and, if possible, synthesize it.

EXPERIMENTAL

The following three methods were utilized for studying the tung oil fractions: ion exchange resin method; petroleum ether—ethyl alcohol method; and hydrolysis method.

Ion Exchange Resin Method

As ion exchange resins, cation exchangers (IR-100, IR-120)[1] and anion exchangers (Dowex 3[2], Permutit A[3]) were used. Taking into consideration that the chemical properties would be different according to the method of obtaining the oil, different kinds of oil were treated with each kind of resin.

Regeneration of Resins.—IR-120.—The resin was thoroughly washed with distilled water, conditioned with three cycles of regeneration with C. P. hydrochloric acid and exhaustion with 20% sodium hydroxide, and finally regenerated with a very large excess of analytical reagent grade hydrochloric acid, washed free of acid with distilled water, and air-dried.

Dowex 3.—A 250-Gm. portion of resin was stirred with 2.5 L. of 5% sodium carbonate for one-half hour, filtered, washed thoroughly with distilled water, and air-dried.

Permutit A.—A 250-Gm. portion of resin was stirred with 2.5 L. of 5% sodium hydroxide for one and one-half hours, filtered, washed thoroughly with distilled water, and air-dried. The reaction of the washings approached neutrality (pH 8.3).

General Procedure.—The method utilized was essentially that described by Davis, *et al.* (11), with the exception that Permutit A was used as the anion exchange resin in lieu of Ionac A. The results were analogous to those reported by the previous workers.

One result, which is unexplainable at the moment, is the failure of Dowex 3 to remove the acidic substances from the oil as did Permutit A.

The results of the use of both the cationic and the anionic resins are summarized in Table I.

Petroleum Ether-Ethyl Alcohol Method

a. A 750-Gm. portion of oil was dissolved in 750 ml. of petroleum ether in a 3-L. separatory funnel and shaken three times with 300 ml. of 80% ethyl alcohol. When the amount of petroleum ether was insufficient, an emulsion occurred on shaking with the alcohol. The alcohol layer was yellow and transparent. An additional 2,600 Gm. of the oil was treated in the same manner. The combined alcoholic extract was allowed to stand in an evaporating dish at room temperature. As the alcohol evaporated, a colorless needle-like crystalline substance was produced on the resinous surface of the liquid. A sample of this crystalline substance was removed; it melted at 40° and became resinous in the air.

The alcoholic extract was completely resinified after a month; it was treated with small amounts of cold alcohol several times. Most of the resin dissolved in the alcohol and a mixture of a white granular material and a colored film remained. The residue was washed with hot ethyl acetate and treated with hot ethyl alcohol several times. Only the colored film (about 0.1 Gm.) remained; it was removed by filtration. From the combined alcoholic filtrate, a white substance, m. p. 265–270°, was obtained by evaporation of the solvent. This was twice recrystallized from 95% ethyl alcohol with the aid of animal charcoal, yielding a white, needle-like crystalline substance, m. p. 295–297°.

On standing at room temperature for a long time, the same crystalline substance was obtained from the alcoholic filtrate of the original extract, from which the mixture of the white granular material and the colored film had been separated. The total yield was 265.1 mg. (0.0071%).

This crystalline substance was quite insoluble in water, 10% sodium hydroxide, and 10% hydrochloric acid. It was very sparingly soluble in alcohol, methanol, ethyl acetate, ether, acetone, benzene, carbon tetrachloride, and petroleum ether; readily soluble in pyridine, dioxane, and amyl alcohol. Its alcoholic solution was not colored with aqueous ferric chloride. Most of the sterol color tests (Salkowski test, Liebermann-Burchard test, and Tschugajeff test) were positive, but the Rosenheim test was negative. It was immediately colored dark red on treatment with concentrated sulfuric acid. Analysis yielded the following results:

Anal.—C, 72.94, 72.67; H, 10.55, 10.39.

Hydrolysis.—The method utilized for this process was that of Power and Salway (12). Eighty-nine milligrams of the substance (m. p. 295–297°) was dissolved in 5.4 ml. of hot *n*-amyl alcohol; 1.8 ml. of an aqueous 15% solution of hydrogen chloride was added together with sufficient ethyl alcohol (7 ml.) to form a homogeneous liquid. After heating for one hour in a reflux apparatus, steam was passed through the mixture to remove the amyl alcohol. A colored solid was produced, removed by filtration, and washed with distilled water. When the solid substance was crystallized from a mixture of ethyl alcohol and ethyl acetate (1:1 by volume), it occurred as glistening leaflets (15 mg.) melting at 133°. This substance gave positive Liebermann-Burchard and Tschugajeff color tests, but was negative in the Rosenheim test. Carbon and hydrogen analyses yielded the following:

Anal.—C, 80.24, 80.49, H, 11.65, 11.43.

The aqueous acid liquid, from which the phytosterol had been separated by filtration, was neutralized with sodium carbonate and evaporated to dryness on the water bath. The residue was digested with absolute ethyl alcohol several times and the mixture filtered. On evaporating the alcoholic fil-

[1] IR-100 and IR-120, products of Rohm & Haas Co., Philadelphia 5, Pa.
[2] Dowex 3, a product of Dow Chemical Co., Midland, Mich.
[3] Permutit A, a product of Permutit Co., 330 W. 42nd St., New York 36, N. Y.

TABLE I.—CATION AND ANION RESIN EXTRACTS FROM TUNG OIL

Experiment No.	Type of Resin	Resin	Oil, Gm.	Extract, Gm.	Acid, mg.
1	Cation	IR-100	3367	0.015	...
2	Cation	IR-100	1272	0.0081	...
3	Cation	IR-120	1464	0.0037	...
4	Cation	IR-120	7213	0.01	...
5	Cation	IR-120	7397	0.027	...
6	Cation	IR-120	17099	0.065	...
7	Cation	IR-120 Regenerated	11320	0.0403	...
8	Anion	Regenerated Dowex 3	2500	0.0096	...
9	Anion	Regenerated Permutit A	12003	8	210.7

trate, a small amount of a syrupy residue was obtained. This had a caramel odor, reduced Fehling's solution and gave a positive Molisch reaction, but no osazone was obtained.

b. A 14,010-Gm. portion of a solvent extracted tung oil was treated according to the general procedure outlined previously. The previous oil sample had been obtained by mechanical expression. The yield of the crystalline substance, m. p. 295–297° was, however, only 6 mg. (0.00005%). The yield of the 80% ethyl alcohol extract was about 90 Gm. This was reserved for pharmacological evaluation.

Hydrolysis Method

A 200-Gm. portion of a tung oil was saponified by refluxing in a 1-L. round-bottom flask with 60 Gm. of potassium hydroxide, 50 ml. of water, and 500 ml. of 95% ethyl alcohol according to the previously described method (4). The flask and its contents were swirled occasionally during the thirty-minute saponification period. After cooling, the soap was acidified in a separatory funnel with 725 ml. of 2 *N* hydrochloric acid. The liberated acids were separated from the aqueous layer and dissolved without further treatment in 1 L. of 95% ethyl alcohol on the water bath. The solution stood for twenty-four hours in the refrigerator. The light colored precipitate was removed by filtration, then washed with about 75 ml. of cold 95% ethyl alcohol. In order to avoid air oxidation of this substance during filtration, the rate of filtration was adjusted so that solvent vapors were always in contact with the crystals. It was recrystallized from 500 ml. of 95% ethyl alcohol; the yield was 75 Gm., m. p. 48°.

The residual yellow aqueous phase, which possibly could contain some water-soluble acidic substances, was neutralized with 20% potassium hydroxide, and evaporated at room temperature. When most of the alcohol had evaporated, the volume of the solution was 950 ml., the solution was yellow and possessed a characteristic odor. This was frozen with dry ice and acetone, and by means of the Campbell-Pressman Apparatus,[4] was subjected *to freeze-drying*. One hundred twenty-nine grams of light colored solid was obtained. Most of this was inorganic in nature. It was treated twice with 100 ml. of cold absolute ethyl alcohol and the combined filtrate was evaporated at room temperature. A yellow syrupy extract (16 Gm.) was obtained. This was reserved for pharmacological evaluation.

[4] This apparatus is produced by E. Machlett & Son Co., 220 East 23rd St., New York 10, N. Y.

DISCUSSION OF RESULTS

Ion Exchange Resin Method.—As may be noted in Table I, in most of the cases, a small amount of extract was obtained except with Permutit A. In the case of Permutit A, a considerable quantity of extract and a small amount of crystals were obtained. It was established that this crystalline material was identical with the 9,14-dihydroxy-10,12-octadecadienoic acid which Davis, *et al.* (11), had isolated from tung oil by the use of an anion exchange resin (Ionac A300).

If this acid had hypotensive activity and is an artifact resulting from autoxidation of eleostearic acid during production and fractionation, as Davis, *et al.* (11), have suggested, conditions for the autoxidation of eleostearic acid in the experiment should be studied for the purpose of obtaining a better yield. Comparison of yield: Davis, *et al.*, 0.0027% (crystals of m. p. 93–98°); present investigation, 0.0018% (m. p. 102–103°).

Petroleum Ether-Ethyl Alcohol Method.—The melting point of the white crystalline substance, m. p. 295–297°, is considerably higher than those of the sterols which were reported by Bilger, *et al.* (8). Indeed, the melting points of the ordinary free phytosterols are mostly less than 200°; ergosterol m. p. 113°, stigmasterol m. p. 169°, fucosterol m. p. 124°, etc.

Some sterol color tests also give positive results with saponins. This substance, however, is different from the saponin which was reported by Emmel (6) for the following reasons: Most of the saponins are soluble in water and elicit characteristic foaming but this substance does not; and generally, saponins are amorphous with the exception of dioscin, sarsasaponin, and cyclamin, but this substance is in the form of microscopical needles.

Elemental analysis of this substance indicated that no nitrogen is present. Accordingly, it is also different from the cyanogenetic glycoside reported by Henry and Auld (5) and the albuminoid reported by Carratolá (7).

It thus appears that this substance is a constituent not previously reported in tung oil.

Presumably, this substance belongs to the phytosterolins (phytosterol glycosides) because of the following properties: (*a*) It elicits a positive sterol color test. (*b*) Its elemental analysis is very similar to that of phytosterolins (12). (*c*) On hydrolysis, this substance yielded a crystalline material, m. p. 133°, which gave a positive sterol color test, and a syrupy substance which reduced Fehling's solution and gave

a positive Molisch reaction. (*d*) It exists as microscopically small needles and its melting point is relatively high and not sharp. These properties are common in phytosterolins. (*e*) Hall and Gisvold (13) obtained simultaneously a free sterol and its glycoside from the same plant. By analogy, it is probable that the glycosides of the free sterols reported by Bilger, *et al.* (8), exist in the tung oil. (*f*) The process of isolation of it is similar to that of phytosterolins. Most of the phytosterolins were isolated from an organic solvent extract; sometimes they result from aqueous extracts by preserving them for a long period of time.

The percentage content of this crystalline substance in various types of tung oil is apparently different. The percentage yield of this substance in different experiments was 0.0071% and 0.00005%, respectively. The amount of this substance obtained from tung oil by the expression method may be more than that obtained by the solvent method, as this substance is quite insoluble in ordinary organic solvents.

The crystalline material, m. p. 40°, may be impure eleostearic acid which was produced during the processing of tung oil, since this crystalline substance resinifies in the air. Eleostearic acids generally give this characteristic behavior.

Hydrolysis Method.—Originally, this method was designed to study the fraction of tung oil not containing eleostearic acid. The freeze-drying techniques were utilized to avoid the decomposition of any heat labile component. Further chemical treatments of the yellow syrupy extract are in progress.

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A Study of LSD-Serotonin Central Interaction*

By PAUL ZANOWIAK† and MORTON J. RODMAN‡

Serotonin pretreatment increases the duration of sleep produced by a variety of central depressants. Lysergic acid diethylamide (LSD) pretreatment inhibits or completely reverses such serotonin potentiation of sleep produced by hexobarbital, mephenesin, and combinations of depressants in which these drugs exert predominant actions. LSD does not suppress serotonin potentiation of hypnosis produced by butabarbital and meprobamate; on the contrary, LSD alone increases the duration of sleep with these compounds to about the same degree that serotonin does.

IT HAS BEEN SUGGESTED that serotonin plays a part in both normal and abnormal mental processes and that the striking psychological effects of lysergic acid diethylamide (LSD) are the result of its ability to interfere with some central action of serotonin (1, 2).

The hypotheses are based in part on the occurrence of serotonin in the brain and the fact that LSD specifically antagonizes the actions of serotonin at a number of peripheral sites *in vitro* (3, 4). More recently, several pharmacological tests have been proposed for demonstrating a similar central antagonism (5, 6, 7).

One such method of evaluating central antiserotonin action is based upon the finding that serotonin prolongs barbiturate hypnosis and that this action is suppressed by prior administration of certain serotonin antagonists. Those properties of serotonin and LSD have been demonstrated in mice, rats, and rabbits depressed by hexobarbital, thiopental, and thialbarbitone (7, 8, 9).

The purpose of this study was to determine whether the results reported in animals receiving such thiobarbiturates could be reproduced when hypnosis was brought about by certain other types of central depressants, administered alone or in various combinations.

METHODS AND MATERIALS

Unfasted male Swiss albino mice from 18–25 Gm. were used only once. Animals were obtained from a single source and kept in the laboratory for at least two days prior to use, to avoid variation. All experiments were carried out during a four-week

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period in the spring of a single year. Room temperature was controlled at approximately 70° F. Thus, differences in sleeping time are not attributable to variation in the environmental temperature.

Control groups were used in all experiments, each animal receiving an injection of saline solution equivalent to the volume of drug solutions employed. The criterion for duration of sleeping time was the time interval between loss and return of the righting reflex. Due to difficulty in determining a firm end point under some circumstances, animals were returned to their backs after apparently spontaneously righting and this was considered a true return of the reflex only when the animal righted itself again within thirty seconds. Standard errors of the mean sleeping times were calculated by the method of Miller and Tainter (10).

All drugs were administered intraperitoneally in the form of freshly prepared solutions. LSD was given in a dose of 10 mg./Kg. one hour before the depressants. Serotonin in a dose of 20 mg./Kg. as serotonin creatinine sulfate, was injected ten minutes before the hypnotics. The depressants were administered in doses which produced a standard degree of central depression, the MED_{50}. This was the dose required to produce hypnosis for a minimum specified period (thirty or sixty minutes) in half of the group of animals. For hexobarbital, and the lower dose levels of mephenesin and meprobamate, the MED_{50} is for thirty minutes; for butabarbital and the larger doses of mephenesin and meprobamate, the MED_{50} is for sixty minutes. The dosages for the combinations consisted of one-half MED_{50} of each barbiturate combined with mephenesin and meprobamate in amounts known to act synergistically with the barbiturates to produce an effect equal to that of one MED_{50} of each barbiturate.

All these doses were derived from an investigation of barbiturate-lissive synergism conducted previously in this laboratory in accordance with the procedure of Gaddum (11), as employed by Berger and Lynes (12). As apparent from an inspection of the various doses in the tables: hexobarbital and mephenesin were additive, butabarbital and mephenesin were incompletely additive, and meprobamate potentiated the depressant action of both barbiturates.

RESULTS

A characteristic sequence of behavioral changes was observed in all animals receiving LSD. The mice became agitated shortly after injection. This was manifested by continual movements of the head from side to side. Often the animals circled rapidly and restlessly about the cage. On other occasions this pattern, which was suggestive of searching, was replaced by one of apparent fear. The animals walked backward in an oddly uncoordinated manner, and, when touched, they would react with strong almost convulsive escape activity. Bristling of the hair and reddening of the ears also occurred frequently. Such excitement began to diminish about thirty minutes afterward and had ceased entirely at the end of one hour, just prior to administration of the depressant. Frequently, the animals would now lie quietly in one position and offer little resistance to the subsequently administered injections.

Serotonin administered by itself ten minutes before the depressant did not produce loss of the righting reflex. It did, however, consistently cause a decrease in spontaneous activity. During this period of slight depression, the mice would lie quietly in one position but were capable of moving when stimulated.

The results of administration of the drugs alone and in various combinations are summarized in Tables I, II, and III. The values stated refer to the mean sleeping times in minutes and the standard error. The figures in parentheses indicate the number of animals used in each series.

The results obtained with hexobarbital, LSD, and serotonin are essentially in accordance with those previously reported in experiments of this type; that is, serotonin potentiated hypnosis produced by the barbiturate and this action was partially suppressed by prior administration of LSD. LSD alone considerably reduced hexobarbital hypnosis, contrary to one reported observation indicating that LSD by itself had no effect on barbiturate sleeping time (13).

The results of the two mephenesin series were likewise in accordance with the results reported previously for barbiturates, except that LSD completely counteracted serotonin potentiation of mephenesin hypnosis, reducing the duration of sleep to a point somewhat below that attained with mephenesin alone at both dose levels.

With butabarbital and meprobamate, on the contrary, the responses to LSD alone and to LSD combinations were quite different from those reported for thiobarbiturates and those observed here with mephenesin. Pretreatment with LSD prolongs the duration of hypnosis produced by these agents in a manner indistinguishable from the potentiating effect of serotonin. Likewise, pretreatment with LSD does not significantly inhibit such serotonin potentiation of the central depressant action of these compounds. This is especially true in the case of butabarbital, in which potentiation by LSD alone, serotonin alone, and combined LSD-serotonin is strikingly similar; with meprobamate some slight suppression of serotonin potentiation is apparent, but the duration of sleep is still nearly double that of the control group at the lower dose level.

Responses of the animals receiving combinations of depressants after pretreatment with LSD and serotonin alone and in combination were comparatively variable. However, the effects on duration of hypnosis appeared, generally, to be related to those which might be predicted in the light of the foregoing findings. That is, potentiation, and suppression of this response appeared to depend largely on the type of depressants employed to produce the basic narcosis. Thus, for example, in animals treated with the hexobarbital-mephenesin combination, the effects after pretreatment were similar to those which had occurred when each of the depressants had been used separately.

With butabarbital-meprobamate, which like hexobarbital-mephenesin is a combination of drugs producing essentially similar responses, the results were also largely as might have been predicted. Failure of serotonin to potentiate hypnosis significantly in this series is a notable exception.

TABLE I.—EFFECTS OF PRETREATMENT OF MICE WITH LSD AND SEROTONIN ON LENGTH OF HYPNOSIS OF RELATIVELY SHORT DURATION

Drug and Dose, mg./Kg.	Depressant Alone, minutes	Depressant plus LSD, minutes	Depressant plus Serotonin, minutes	Depressant plus LSD Plus Serotonin, minutes
Hexobarbital 100	28.0 ± 1.3 (50)[a]	17.3 ± 1.0 (10)	53.2 ± 1.4 (19)	38.0 ± 2.4 (10)
Mephenesin 320	28.0 ± 1.2 (43)	16.3 ± 1.0 (10)	42.1 ± 1.9 (20)	24.4 ± 1.3 (10)
Meprobamate 240	44.9 ± 2.3 (30)	84.7 ± 5.3 (10)	97.5 ± 1.8 (20)	86.6 ± 1.8 (10)

[a] The figures in parentheses indicate the number of animals used.

TABLE II.—EFFECTS OF PRETREATMENT OF MICE WITH LSD AND SEROTONIN ON LENGTH OF HYPNOSIS OF MODERATELY LONG DURATION

Drug and Dose, mg./Kg.	Depressant Alone, minutes	Depressant plus LSD, minutes	Depressant plus Serotonin, minutes	Depressant plus LSD plus Serotonin, minutes
Butabarbital 56	75.8 ± 2.7 (60)[a]	153.0 ± 7.7 (20)	146.0 ± 5.5 (19)	154.9 ± 7.7 (10)
Meprobamate 280	70.8 ± 4.4 (38)	131.0 ± 7.5 (10)	127.6 ± 4.0 (18)	107.0 ± 3.6 (10)
Mephenesin 435	76.7 ± 1.1 (30)	45.1 ± 1.4 (10)	125.6 ± 1.7 (20)	46.9 ± 3.6 (10)

[a] The figures in parentheses indicate the number of animals used.

TABLE III.—EFFECTS OF PRETREATMENT OF MICE WITH LSD AND SEROTONIN ON LENGTH OF HYPNOSIS PRODUCED BY COMBINED DEPRESSANTS

Drug Combination and Doses, mg./Kg.	Depressant Alone, minutes	Depressant plus LSD, minutes	Depressant plus Serotonin, minutes	Depressant plus LSD plus Serotonin, minutes
Hexobarbital 50; Mephenesin 160	27.8 ± 1.5 (30)[a]	23.2 ± 1.0 (10)	64.0 ± 3.7 (20)	27.0 ± 1.9 (10)
Hexobarbital 50; Meprobamate 72	22.3 ± 0.9 (30)	34.3 ± 2.6 (10)	34.6 ± 3.3 (20)	25.0 ± 1.6 (10)
Butabarbital 28; Mephenesin 300	62.6 ± 2.2 (30)	53.7 ± 2.9 (20)	158.0 ± 5.6 (20)	82.6 ± 4.2 (10)
Butabarbital 28; Meprobamate 67	58.9 ± 1.1 (30)	91.2 ± 3.7 (10)	65.4 ± 2.7 (20)	118.0 ± 7.5 (10)

[a] The figures in parentheses indicate the number of animals used.

In the series in which combinations of differently acting depressants were used, the data are most variable. However, this could be expected, due to the different responses elicited by each drug administered alone after LSD and serotonin pretreatments. Generally, the duration of sleeping time tended to be influenced by the predominant drug in each mixture. Thus, for example, the partial suppression by LSD of serotonin potentiation of hypnosis in both series may be due to the predominance in each case of the drug, which, when used alone, elicits a similar response. In the hexobarbital-meprobamate combination, this is the barbiturate; in the butabarbital-mephenesin combination, the effects of the latter drug predominate. In each combination, the drug exerting the more influential effect is the one which is present as a greater fraction of its MED_{50}, and which, administered alone, would cause a longer depression.

DISCUSSION

Despite a paucity of direct evidence that serotonin takes part in normal brain function at all, it has been suggested that an abnormality in central serotonin function may be the basis of certain mental disturbances (14–16). According to these views, mental illness may be the result of a metabolic function resulting in the development of either a functional deficiency or excess of serotonin at the central sites at which it performs certain postulated physiological functions.

Because of the remarkable potency of LSD in producing symptoms similar to some seen in schizophrenia and its antagonism of certain peripheral effects of serotonin this synthetic ergot alkaloid and its congeners have been widely employed as pharmacological tools in the investigation of these various working hypotheses (6, 7, 17). Data obtained in such studies, while often meager and indirect, have been interpreted in terms that favor one or another of the current biochemical concepts of mental disorder.

While we are unwilling to attempt an interpretation of our own equally indirect results in terms of the complex problem of whether serotonin plays a part in normal and abnormal mental processes, it may be profitable to consider the data in relation to the more limited problem of whether the psychological effects of LSD are related to a central interaction with serotonin, as has been suggested.

Data, such as those reported here for hexobarbital-serotonin-LSD interactions, have been offered as evidence for the concept that LSD suppressed some central action of serotonin (7). It has been suggested that such suppression of serotonin potentiation of hexabarbital hypnosis is the result of replacement of serotonin and its action at receptors by LSD (13). The fact that LSD by itself had little effect on hypnosis produced by the barbiturate has been explained in terms of the antimetabolite concept. That is, LSD, a structural analog of serotonin, is believed to act as an antiserotonin, because it is sufficiently close to serotonin in chemical configuration to combine with its receptors without,

however, being able to fulfill the physiological role of the hormone (1, 5, 15, 16).

However, this concept of a central antagonism between LSD and serotonin is not borne out by the results of our experiments, in which butabarbital and meprobamate, rather than hexobarbital, are the basic depressants. Here, not only does LSD fail to reverse serotonin potentiation of hypnosis, but LSD by itself prolongs the duration of hypnosis to an extent at least equal to the effect of serotonin alone on the action of these depressants. Thus, in these experiments, LSD appears to produce a "serotoninlike" response. This may mean that LSD acts, not by creating a functional deficiency of serotonin, but an excess.

The view that LSD may mimic the action of serotonin rather than compete with it is supported by a number of other observations in which LSD had an effect similar to that of serotonin (18). Depending upon the test object and concentration of LSD employed, both antiserotonin and serotoninlike results have been reported. Wooley and Shaw (18) have attempted to account for these actions by suggesting that serotonin receptors are not all alike but vary from tissue to tissue. LSD may then be pictured as fitting some of these receptors well enough, not only to block the effects of serotonin, but to itself induce a serotoninlike effect.

Our data especially for the butabarbital series, offer some support for this concept of LSD combining with serotonin receptors well enough to act like the hormone itself. There is little difference in the degree to which LSD alone and serotonin alone potentiate butabarbital hypnosis. Furthermore, pretreatment with both LSD and serotonin produces a potentiation of butabarbital hypnosis that differs very little from that caused by pretreatment with either substance alone. This failure of serotonin to increase duration of butabarbital sleep beyond that brought about by LSD alone may mean that the hormone cannot combine with receptors already occupied by LSD.

While the antimetabolite theory may be invoked to account for both the antiserotonin and serotoninlike actions of LSD in these experiments, one crucial question remains unanswered. What properties of the drugs themselves account for the differences in their interactions with LSD and serotonin?

One property which must be considered is the difference in duration of action of these depressants. Because butabarbital and meprobamate are classified as relatively long in action compared to hexobarbital and mephenesin, it might be argued that the difference in response to the two groups after LSD pretreatment may be due to a temporal factor. Thus, for example, early LSD stimulation might be followed by a late depressant action which would synergize with that of the longer acting drugs; with the short acting compounds, on the other hand, recovery could occur before this delayed depressant action of LSD developed.

That such a temporal relationship is not involved, however, is indicated by the results of a number of experiments where LSD and the barbiturates were administered simultaneously. The effects of LSD on the duration of hypnosis were nearly identical with those produced when it was given one hour prior to the barbiturate, hexobarbital sleeping time was reduced and butabarbital hypnosis nearly doubled.

Evidence that the differences in the interactions of the depressants with LSD are independent of the duration of action of the drugs is also seen in the results of the mephenesin series. Mephenesin, whether employed in doses that cause sleep of long or short duration, produces a characteristic response quite different from that of butabarbital and meprobamate, when all three drugs are given in equipotent doses in animals pretreated with LSD and serotonin (Table II).

Differences in interactions of different drugs with LSD and serotonin might also be accounted for in terms of their varying sites and mechanisms of action. Thus, the response to a drug acting at cells possessing one type of serotonin receptor might well be different from that of a drug acting at sites containing receptors responsive to both serotonin and LSD. Or certain drugs, such as butabarbital and meprobamate, might act to sensitize serotonin receptors or otherwise change the conditions there, with the result that previously inactive LSD might be activated and produce a serotoninlike effect. However, the results obtained here do not support such speculation, at least not in the present state of knowledge of the sites of action of these drugs. On the contrary, it is noteworthy that differences in interaction with LSD and serotonin occur between drugs of the same general classifications.

While the observations reported here are not readily explained in terms of current concepts of central serotonin function, they appear to reduce the plausibility of the idea that the psychological effects of LSD result from a simple antagonism between it and serotonin, such as seems to occur peripherally *in vitro*.

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Prediction of Stability in Pharmaceutical Preparations[1] V*

The Solution Degradation of the Antibiotic Streptovaricin[2]

By EDWARD R. GARRETT

The prediction of stability and pharmaceutical formulation of compounds of unknown structure are especially important in the antibiotic age. The kinetics of solution degradation of the antibiotic complex of the structurally unknown streptovaricins are studied spectrophotometrically and equations obtained to characterize degradation as a function of pH. Bioassay has been correlated with spectra and conditions given for maximum stability of assay standards and preservation of yield. A mechanism is proposed that accounts for the available data and predicts the properties of degradation intermediates. The application of an analog computer to kinetic interpretation is given. Kinetic conditions for the differential assay of streptovaricin C were derived.

THERE IS a need in pharmacy to study the changes in structures of molecules of unknown structure.

This apparently contradictory circumstance has become more evident with the advent of the antibiotic age. The application, efficacy, and toxicity of an antibiotic or antibiotic complex is frequently well known before the components can be separated, purified, individually characterized, and structures assigned. Pharmacy is often called upon to apply its unique talents in formulation and stabilization before the assignment of structure, perhaps even before ultimate purification.

The stability of materials must be known for the establishment of treatment of assay standards and for the preparation of samples to be submitted for clinical evaluation. Yet no knowhow may be available as to what part of the molecule is hydrolyzed, pyrolyzed, or photolyzed; or how such degradations may be easily and quantitatively followed.

In general, the primary criteria of efficacy for compounds with such histories will be bioassays. However, bioassays as criteria of stability involve considerable expense, large error, and special isolation techniques that are frequently tedious and complex.

Prediction of stability and design of formulations may be based on studies of the rates of change of some physicochemical characteristic. Correlation of changes in the physicochemical properties with the changes in the bioassays may serve as a valid argument for the use of the former. An obvious disadvantage is that variation in the latter may not necessarily reflect in the former although the converse is not probable.

Obvious advantages are that such physicochemical studies will be easy to conduct, less expensive, can lead to prediction of the conditions that affect degradation and provide information on the properties of the intermediates and products. These facts can contribute to process design for maximum yield, the achievement of stable standards, and the least degrading assay conditions.

Examples of the application of such a philosophy were the stability studies on the antibiotic fumagillin (2, 3, 4).

The antibiotic complex of the streptovaricins of unknown structure is a good case in point. The complex is of particular interest because of its high antitubercular activity (5, 6, 7), yet it is a complex of closely related antibiotics with no apparently distinguishable ultraviolet spectra and only slightly variable infrared spectral differences (8). The streptovaricin complex consists of at least five components, A, B, C, D, and E (5, 8), separable by the tedious processes of paper chromatography (9) and countercurrent extraction (8).

This paper reports on studies designed to determine the total and comparative stabilities of the streptovaricin components as a function of pH, to predict the nature of the intermediates or products of degradation from the physicochemical changes, to provide clues for easier separation or easier assay by differential degradation, and to predict the conditions for maximum stability in solution.

EXPERIMENTAL

Four components of the streptovaricin complex, i.e., A, B, C, and D, were isolated by chromato-

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The author is greatly indebted to Mrs. Lillian G. Snyder for excellent technical assistance, to Dr. W. T. Sokolski and associates for the microbiological assays, and to Mr. Clayton D. Alway for construction of and instruction on the analog computer.

[1] See reference (1) for prior paper in this series.

[2] The trade name of The Upjohn Co. for the antimicrobial streptovaricin is Dalacin.

graphic column and subsequent Craig countercurrent distribution according to the procedure of Herr, *et al.* (8). The compounds used were prepared by these authors.

The general procedure for the ultraviolet spectrophotometric studies on the solution degradation of the streptovaricins was to weigh out the necessary amount of material (80 mg.) and take up to volume in a 500-ml. volumetric flask with absolute ethanol maintained in a 30.3° constant temperature bath. For each buffer solution, 25 ml. of the ethanolic solution was pipetted into a 100-ml. volumetric flask and brought up to volume with the appropriate buffer, previously equilibrated at the constant temperature. The final concentration was 40 μg./ml. in the streptovaricin and the final buffer concentration is given in Table I.

The resultant solutions were run at various intervals on the Cary recording ultraviolet spectrophotometer, Model 11, against a 25% ethanol-buffer blank made up at the same time as the streptovaricin solutions.

The pH values were checked immediately and at intervals on a Cambridge pH meter, equipped with glass-saturated calomel electrodes. The pH values were consistent throughout the runs and averages are given in Table I.

Spectrophotometric readings were also made on the streptovaricin solutions in absolute ethanol and on nitrogen purged, buffered aqueous ethanolic solutions. No significant spectral change was observed in the former case and nitrogen purging had no retarding effects on the degradation rates in the latter case.

Material submitted for bioassay against *S. lutea* (9, 10, 11) was diluted to the appropriate concentration range. When the degradation was in the highly alkaline or acidic regions, the samples taken for bioassay were immediately neutralized so as to stabilize the streptovaricins submitted.

In order to study the effects of varying salt concentrations and check the possibilities of general acid-base catalysis the pH was maintained constant but the acetate-acetic acid buffer concentration was varied. (See Trials 11, 12, and 13 in Table I). No significant change in degradation rate was observed.

RESULTS

Streptovaricin C Degradation as Characterized by the 432 and 316 mμ Chromophores.—The ultraviolet spectra of streptovaricin are given by Curve A, Fig. 1. The spectra are independent of pH within the range 1.0–9.0. Representative wavelengths characteristic of the spectrum are the 432 mμ maximum, 316 mμ plateau, the shoulder maximum at 263 mμ, and the major maximum at 244.5 mμ.

Within the pH range 1.0–9.0, the absorptivity at the 432 mμ maximum decreases with time to an asymptotic value at apparently the same rate as does the absorptivity at the 316 mμ plateau. The rate constants of the apparent first order degradation may be determined by application of the expression to the absorbance:

$$\log (A - A_\infty) = -kt/2.303 + \log (A_o - A_\infty) \qquad \text{(Eq. 1)}$$

where A_o is 0.600 at 432 mμ and 0.615 at 316 mμ, and

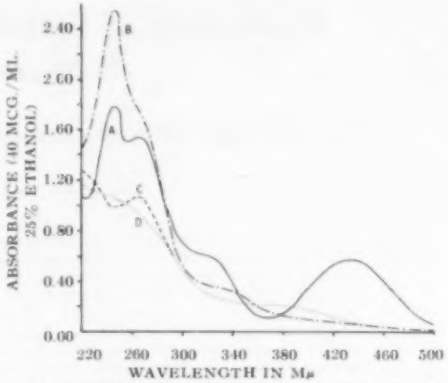

Fig. 1.—The ultraviolet spectra of streptovaricin C and degradation products. Curve A.—all streptovaricins; Curve B.—first product of degradation in acid solution, i.e., streptovaricin C′ in 0.1 *M* HCl; Curve C.—final product of acid degradation and acidified neutral degraded material, i. e., streptovaricin C″ in 0.1 *M* HCl solution; Curve D.—final product of neutral degradation and neutralized acid degraded material, i. e., streptovaricin C″ at pH 7.4.

where A_∞ is 0.100 at 432 mμ and 0.320 at 316 mμ for 40 μg. streptovaricin C per ml. of 25% ethanol. The given A_∞ at 316 mμ is only valid above pH 2.00. The absorbance, A, is at a time, t.

Apparent first order plots in accordance with Eq. 1 for absorbance changes at 432 mμ at representative pH values are given in Fig. 2. The estimated rate constants (k in sec.$^{-1}$) are given in Table I and a plot of log k *vs.* observed or actual pH is given in Fig. 3. Figure 3 clearly shows that the rate of degradation of streptovaricin C as characterized by the 432 mμ chromophore disappearance is hydrogen ion dependent below pH 3 and a good estimate of the rate (k in sec.$^{-1}$) below pH 3 is:

$$\log k = -\text{pH} - 3.400 \qquad \text{(Eq. 2)}$$

Thus, the apparent mechanism of degradation of streptovaricin C from 432 and 316 mμ observation is:

$$C \xrightarrow{k_H{}^+ [H^+]} C' \qquad \text{(Eq. 3)}$$

where the absorptivities of C are $a_{432} = 15{,}000$, $a_{316} = 15{,}600$, and of C' are $a'_{432} = 2{,}500$, $a'_{316} = 8{,}000$.

If the data on varying HCl molarity is considered (trials 1–7; Table I), then

$$k = 3.27 \cdot 10^{-4}[H^+] + 11.8 \cdot 10^{-7} \qquad \text{(Eq. 4)}$$

This implies that a "water" or spontaneous reaction rate constant, k_o, is $11.8 \cdot 10^{-7}$ sec.$^{-1}$ which is characterized by the horizontal solid line in Fig. 3. The increased rate above this solid line above pH 7 could be attributed to some hydroxyl ion attack; the lower rate at pH 3.25 is difficult to interpret and may or may not be real. The asymptotic value at 316 mμ appears to be a minor function of the pH of degradation and/or the pH of the media (see Curves C and D in Fig. 1). Since a mean A_∞ was assumed constant for this wavelength, less emphasis should be placed

TABLE I.—APPARENT FIRST ORDER RATE CONSTANTS (k IN SEC.$^{-1}$) FOR THE LOSS OF THE 432 AND 316 mμ CHROMOPHORES OF STREPTOVARICIN C[a]

Trial	pH	Buffer Composition	10^5 k (sec.$^{-1}$) 432 mμ	316 mμ
1	1.19	0.075 *M* HCl	243	..
2	1.30	0.060 *M* HCl	207	..
3	1.47	0.040 *M* HCl	144	..
4	1.77	0.020 *M* HCl	77.8	..
5	2.04	0.010 *M* HCl	44.2	..
6	1.12	0.0375 *M* KCl, 0.0783 *M* HCl	258	119
7	1.97	0.375 *M* KCl, 0.0125 *M* HCl	48.2	30.7
8	2.73	0.427 *M* CH_3COOH	9.80	7.57
9	3.24	0.0427 *M* CH_3COOH	5.60	4.65
10	4.38	0.1000 *M* CH_3COOH, 0.025 *M* CH_3COONa	10.5	10.8
11	4.99	0.0264 *M* CH_3COOH, 0.02425 *M* CH_3COONa	12.8	14.3
12	4.99	0.0532 *M* CH_3COOH, 0.0500 *M* CH_3COONa	13.5	15.7
13	4.98	0.1063 *M* CH_3COOH, 0.1000 *M* CH_3COONa	12.8	14.7
14	5.33	0.123 *M* CH_3COOH, 0.4000 *M* CH_3COONa	12.3	13.2
15	5.41	0.0244 *M* CH_3COOH, 0.0628 *M* CH_3COONa	13.1	14.4
16	5.88	0.0210 *M* CH_3COOH, 0.2000 *M* CH_3COONa	8–12	10.0
17	6.22	0.0030 *M* CH_3COOH, 0.0628 *M* CH_3COONa	10–12	9.96
18	7.42	0.0948 *M* KH_2PO_4, 0.0568 *M* NaOH	15.1	9.19
19	8.57	0.0777 *M* KH_2PO_4, 0.0745 *M* NaOH	15–19	11.6
20	7.08	43.8% ethanol, no buffer	4.21	4.51

[a] At 40 μg./ml. in 25% ethanol at 30.3°.

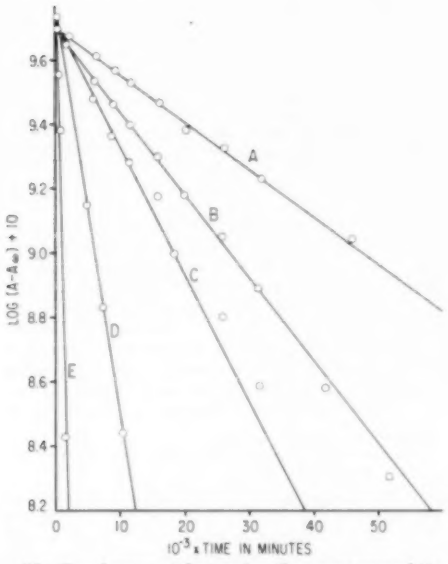

Fig. 2.—Apparent first order disappearance of the 432 mμ absorbance for streptovaricin C at 40 μg./ml. of 25% ethanol at 30.3° as studied at varying pH values. Curve A.—pH 3.24; Curve B.—pH 2.73; Curve C.—pH 7.42; Curve D.—pH 1.97; Curve E.—pH 1.12.

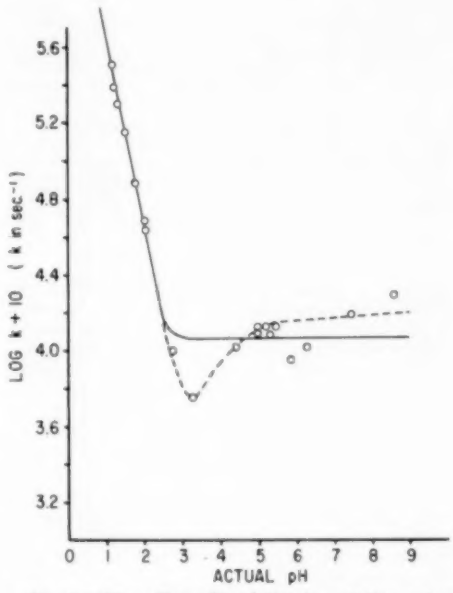

Fig. 3.—The pH profile of the degradation rates of streptovaricin C based on the 432 mμ absorbance disappearance in 25% ethanol at 30.3°.

on the reliability of the rate constants calculated at 316 mμ. Comparison of rates of degradation of nitrogen purged and nonpurged solutions showed no differences. This indicates that air oxidative processes under these conditions were not responsible.

Streptovaricin C Degradation Characterized by Lower Wavelength Chromophores.—Streptovaricin C at low pH values shows an increase and then a subsequent decrease in absorbance at the low wavelengths, i.e., at 245 mμ and 263 mμ. The rate of increase at 245 mμ corresponds to the rate of decrease at 432 mμ. Figure 4 graphically shows this to be so in that the apparent maximum at 235 mμ correlates with the maximum loss in absorptivity at 432 mμ for pH values below 3.

The spectra of acid degraded streptovaricin C at approximately the time that the maximum absorbance was reached at 245 mμ is shown in Fig. 1, Curve B, and in Fig. 5, Curve A. The latter was obtained after twenty hours at 30° in 0.075 *M* HCl in 25% ethanol at 40 μg./ml. The absorbance is at 12.6 μg./ml. in 25% ethanol at pH 0.8. This may be an isolable intermediate, *C'*, where the increased ab-

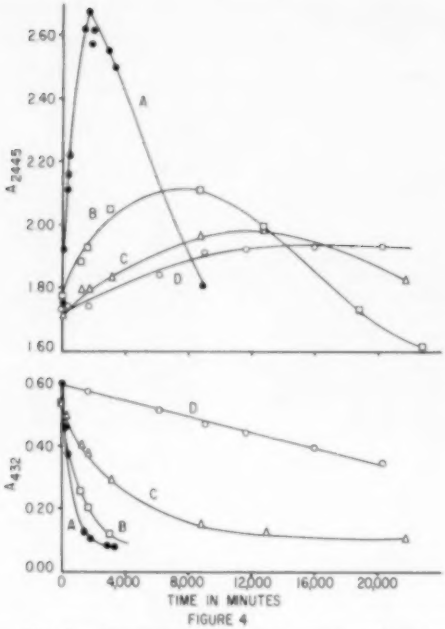

FIGURE 4

Fig. 4.—Demonstration of the correspondence in time of the absorbance, A_{245}, increase to a maximum at 245 mμ and the absorbance, A_{432}, decrease to a minimum at 432 mμ of streptovaricin C at 40 μg./ml. 25% ethanol at 30.3.° Curve A.—pH 1.12; Curve B.—pH 1.47; Curve C.—pH 2.04; Curve D.—pH 3.24.

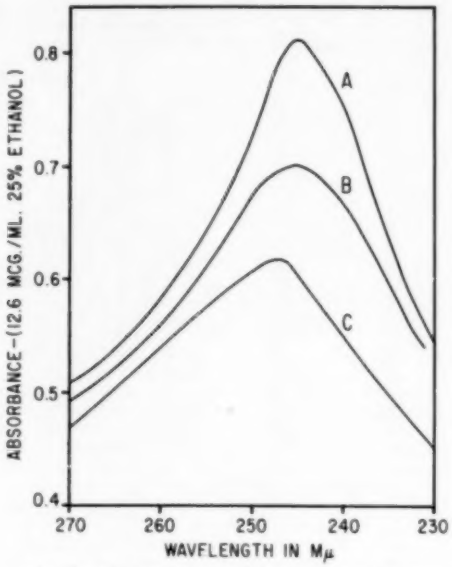

Fig. 5.—Base-acid reversible spectra of streptovaricin C′, an intermediate in streptovaricin C acid degradation. Curve A.—streptovaricin C′ and reacidified neutralized and buffered *C′*; Curve B.—*C′* from acid degradation adjusted to pH 4.33; Curve C.—*C′* from acid degradation adjusted to pH values of 5.4, 6.1, 7.4, and 8.2.

sorbance at 245 mμ washes out the still-present subsidiary maximum at 263 mμ. The latter absorption in Fig. 1, Curve B, does not appear to be significantly altered in intrinsic intensity by the transformation.

The intermediate, *C′*, exhibited a decrease in absorbance of the 244.5 mμ when adjusted to pH values in excess of 2.5. Curve A, Fig. 5, is not only the spectra of *C′* but of *C′* obtained from neutral degradations adjusted to pH values of 1.2 and 2.4. Curve B, Fig. 5, is the spectra of *C′* adjusted to pH 4.33 whereas Curve C is adjusted to pH's of 5.40, 6.05, 7.39, and 8.24 by sodium hydroxide and acetate buffers. Curve A is also the result when these latter solutions are acidified with HCl to obtain the original acid conditions.

These facts permit the conclusion that the acid degradation to *C′* has produced a structure with chromophoric enhancement at 245 mμ and chromophoric loss at 432 mμ. The base-acid reversibility of the *C′* chromophore changes at 245 mμ can be attributed to a *C′* functional group of pKa *ca.* 4.

The intermediate, *C′*, however, exhibited a decrease in the 245 mμ absorbance and increases in 263 mμ and 360 mμ absorbances, as well as a visible color when adjusted to pH values in excess of 10. The color cleared on reacidification, but the 245 mμ absorbance was slightly higher than its original value. This indicates a pH susceptible group in *C′* of pKa *ca.* 10 which, in part, is irreversibly affected.

The subsequent decrease in absorbance at 245 mμ after the maximum absorbance (corresponding to *C′*) at low pH values tends to give a final product (Curve C in Fig. 1) with a slight maximum at 263 mμ. This absorbance decrease is apparently subsequent to the elimination of the 432 mμ chromophore and, as will be shown later, is subsequent to the destruction of the bioactivity. Figure 6 shows the absorbance at 245 mμ against time as a function of pH of degradation. The absorbance of the maxima is less when the reaction is studied at higher pH values. Above pH 4 no maximum is observable in such plots.

The spectra of completely degraded *C* does approach asymptotic values. In the neutral pH region the asymptotic spectra has a mild maximum at 237 mμ (See Curve D in Fig. 1). When acidified, this mild maximum is lost but one is gained at 263 mμ which very closely approximates the asymptotic spectra of acid degraded *C* (See Curve C in Fig. 1). Conversely, acid degraded materials (Curve C) when neutralized have spectra closely approximating the asymptotic spectra of neutral degraded material (Curve D in Fig. 1). They are not exactly superimposable but the major chromophores are retained.

Chromophoric Degradation of Streptovaricins A, B, C, and D.—The ultraviolet spectra of undegraded streptovaricin C is independent of pH and the spectra of all streptovaricins are similar except for a small displacement in absorptivity which could be attributed to slight impurities.

The rates of degradation of streptovaricins A. B, C, and D were compared at pH 1.1 and 8.5 as measured at 245, 260, 316, and 432 mμ, all at 30.3° at 40 μg./ml. and in 25% ethanol.

At pH 1.1, the previously discussed initial in-

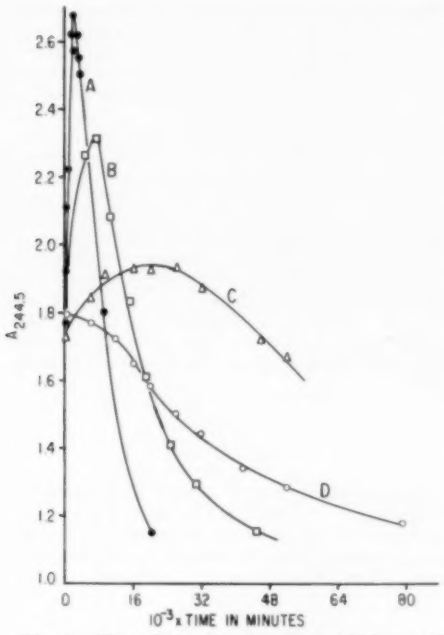

Fig. 6.—Plot of the absorbance at 244.5 mμ of streptovaricin C with time at various pH values. Curve A.—pH 1.12; Curve B.—pH 1.97; Curve C.—pH 3.24; Curve D.—pH 4.38.

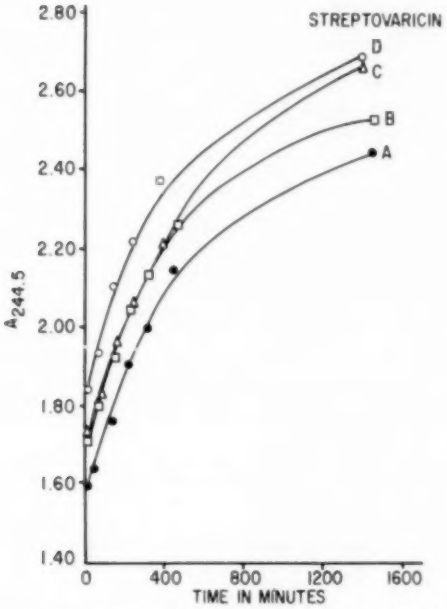

Fig. 7.—Spectral absorbance increase to a maximum with time at 244.5 mμ for various streptovaricins at pH 1.2 in 25% ethanol with HCl-KCl buffer.

crease and subsequent decrease in absorbance at 244.5 mμ and 260 mμ were noted. Except for the expected vertical displacement, the plots of $A_{244.5}$ *vs.* time for the various streptovaricins were parallel (See Fig. 7). The first order plots of 432 and 316 mμ absorbance disappearance showed no gross difference in rate (See Fig. 8) except for a slightly faster degradation rate for streptovaricin D.

At pH 8.5, the same conclusions of similar chromophoric degradation could be made.

Correlation of Bioactivity and Chromophore Degradations.—The concentration of degrading streptovaricin, $[S]$, in 25% ethanol may be computed from the absorbance at any time (A_t) at 432 mμ, the initial absorbance (A_o) of the initial concentration of streptovaricin, $[S]_o$, and the asymptotic absorbance (A_∞) of the degradation product S' where $[S']_\infty = [S]_o$:

$$[S] = \frac{A_t - a'[S]_o}{a - a'} \qquad \text{(Eq. 5)}$$

where $a = A_o/[S]_o$ is the absorptivity of the streptovaricin and $a' = A_\infty/[S]_o$ is the absorptivity of the final product $[S']_\infty$. For streptovaricin C, a = 15,000; a' = 2,500 at 432 mμ.

Streptovaricins in Acid.—The streptovaricin C concentration degraded in 0.075 *M* HCl and as calculated from Eq. 5 at 432 mμ is plotted against the bioassay results in Fig. 9. The correlation, in gen-

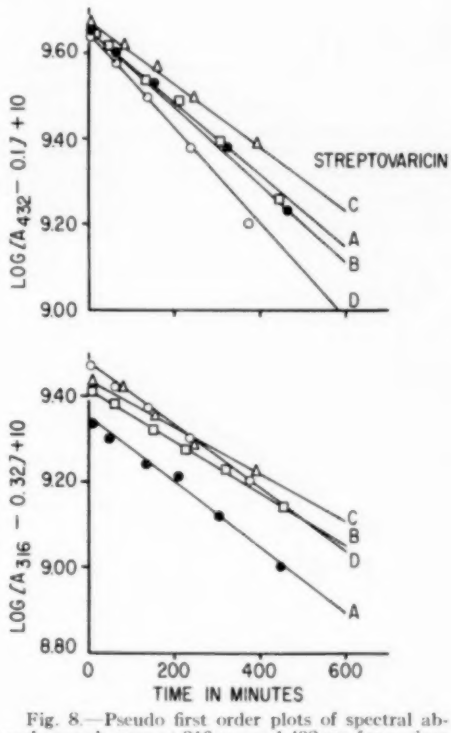

Fig. 8.—Pseudo first order plots of spectral absorbance changes at 316 mμ and 432 mμ for various streptovaricins at pH 1.2 in 25% ethanol with HCl-KCl buffer.

eral, is good. The aliquots submitted for bioassay were adjusted to pH 3–4 after sampling since degradation rate appeared to be lowest in this pH range.

Similar correlations were possible for streptovaricins A and B when these were subjected to acid degradation, i.e., at pH 1.0. However, streptovaricin D demonstrated a faster decrease in 432 mμ chromophore than in apparent activity.

Streptovaricins at Neutral pH.—Streptovaricin C degradation at pH 5.00 (Fig. 10) and similarly at pH 8.5 showed good correlation of bioactivity with concentration calculated from the 432 mμ chromophore. At pH 8.5, however, the other streptovaricins acted anomalously and showed gross deterioration in bioactivity for little change in spectra, completely losing their potency by bioassay within a maximum of eight hours; whereas streptovaricin C only lost 6% as calculated from the rate constants of Table I.

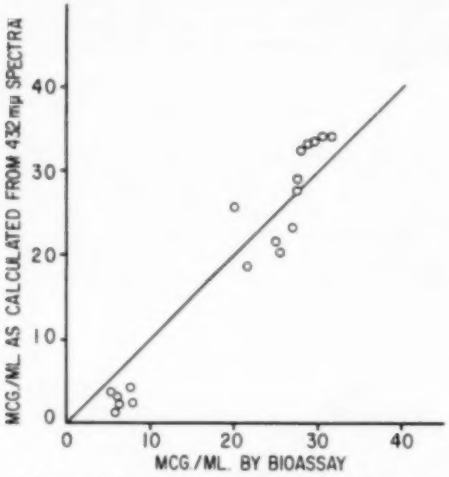

Fig. 9.—Correlation of streptovaricin C concentration in 25% ethanol calculated from 432 mμ absorbance and concentration based on bioassay. The samples were taken at various times in 0.075 *M* HCl and adjusted to pH 4 on sampling. The line represents the theoretical relation.

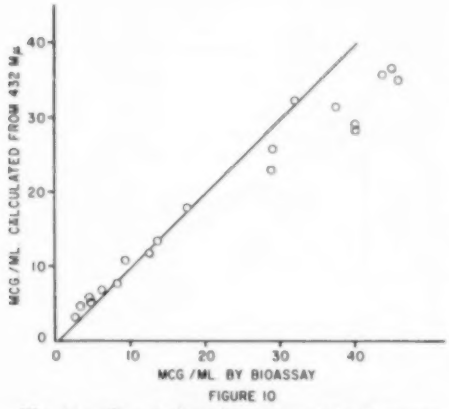

FIGURE 10

Fig. 10.—Correlation of streptovaricin C concentration in 25% ethanol calculated from 432 mμ absorbance and concentration based on bioassay. The samples were taken at various times in pH 5.0 acetate-acetic acid buffer. The solid line represents the theoretical relation.

Streptovaricins in Alkali.—Degradation of streptovaricin C in 0.01 *M* to 0.06 *M* NaOH results in a definite portion of the initial absorbance being destroyed almost instantaneously, *ca.* 40% at 244.5 and 263 mμ, and *ca.* 20% at 316 and 432 mμ. Thereafter, the change in absorbance is extremely slow.

The studies of change in bioassay of streptovaricins A, B, C, and D in 0.01 *M* NaOH were conducted by neutralizing an aliquot with 0.01 *M* HCl and buffering at pH 3. The biological activity was completely gone within fifteen minutes. The estimated half-life of this activity was five minutes at this alkalinity.

DISCUSSION

Possible Mechanism to Explain Streptovaricin C Degradation.—A postulated reaction sequence with equilibria that fit the results from the spectrophotometric studies of the acid and neutral degradation of streptovaricin C are:

$$C \xrightarrow{k = k_H{}^+[H^+] + k_o} \left\{ +H^+ \rightleftharpoons \begin{matrix} C'_1 \\ C'_2 \end{matrix} -H^+ \right\} \xrightarrow{k'} C'' \qquad \text{(Eq. 6)}$$

where the evaluated constants for k are given in Eq. 4 and $C' = C'_1 + C'_2$.

This scheme was substantiated by the following kinetic treatment of the experimental data.

A series of degradation studies of 40 μg. streptovaricin C per ml. of 25% ethanol were conducted by following the absorbance changes with time at 245 mμ. These data which included the examples given in Fig. 6 were analyzed by determining the first order rate, k', of approach to an asymptotic absorbance after the appearance of the maximum absorbance at this wavelength, i. e., for the postulated $C' \rightarrow C''$ transformation. A value for 1.0 for A_∞ was reasonably constant for all studies and the k' in sec.$^{-1}$ were determined from plots of log $(A - A_\infty)$ *vs.* time as per Eq. 1. The k' values so determined are listed in Table II as well as the k values determined from the complete loss of the 432 mμ chromophore in the same study. The rate constant k in sec.$^{-1}$ for the postulated $C \rightarrow C'$ transformation is also considered as the rate constant characterizing the appearance of the 245 mμ maximum observed at the lower pH values since the parallelism of the two spectral changes with time was so apparent.

The total absorbance, A_t, at 245 mμ at any time is made up of the contributions from the nondegraded streptovaricin C, the intermediate C' either in the protonated form, C'_1, or the nonprotonated form C'_2, and the final product C'' as based on Eq. 6. Also, where $(C)_o$ is the initial concentration of streptovaricin C:

$$[C]_o = [C] + [C'] + [C''] \qquad \text{(Eq. 7a)}$$

$$[C'] = [C_1'] + [C_2'] \qquad \text{(Eq. 7b)}$$

The respective absorptivities are a, a', a'_1, a'_2, and a'' so that

$$A_t = A + A' + A'' \quad \text{(Eq. 8a)}$$

$$A_t = a[C] + a'[C'] = (a_1'[C_1'] + a_2[C_2']) + a''[C''] \quad \text{(Eq. 8b)}$$

$$A' = a_1'[C_1'] + a_2'[C_2'] \quad \text{(Eq. 9)}$$

If the equilibrium between C'_1 and C'_2 is hydrogen ion dependent, then

$$[C_2'] = [C_1']K'/[H^+] \quad \text{(Eq. 10)}$$

Thus, substitution of Eq. 10 into Eqs. 7b, and 9 and division of Eq. 9 by 7b result in an equation for the apparent absorptivity, a', of C':

$$a' = A'/[C'] = \frac{a_1' + a_2'K'/[H^+]}{1 + K'/[H^+]} \quad \text{(Eq. 11)}$$

It follows that, at high pH or low $[H^+]$,

$$a' \rightarrow a_1'[H^+]/K' + a_2' \rightarrow a_2' \quad \text{(Eq. 12)}$$

and that, at low pH or high $[H^+]$,

$$a' \rightarrow a_1' + a_2K'/[H^+] \rightarrow a_1' \quad \text{(Eq. 13)}$$

so that the apparent absorptivity, a', varies between a_1' and a_2' and at equivalent amounts of C_1' and C_2' where, in general, $[H^+] = K'$ from Eq. 10, then

$$a' = (a_1' + a_2')/2; \ [H^+] = K' \quad \text{(Eq. 14)}$$

The absorptivities of C and C'' can be determined from the initial and final spectral absorbances at 245 mμ, i. e., $a_{245} = A_o/[C]_o = 46{,}000$ and $a''_{245} = A_\infty/[C]_o = 26{,}000$. The k and k' values for a given pH are also known since the former is the pseudo first order rate constant for the disappearance of the 432 mμ maximum and equivalent to the rate of appearance of the 245 mμ maximum absorbance which appears as an intermediate in that chromophore's change with time.

The curves of 245 mμ absorbance against time as given in Fig. 6 can be fitted by an electrical analog wherein the model is based on Eqs. 6–9. The potentiometer settings to give the best fit can be translated into k, k', a, a', and a'' values. The results of this curve fitting by the use of an analog computer are given in Table II.

Since the manipulation of one potentiometer setting necessitated changes in all the others, this fitting was not an easy procedure. Thus, the striking correlation in Table I of the analogously computed estimates of k (the rate constant for the appearance of C' as a maximum absorbance at 245 mμ) and those determined graphically from the pseudo first order decrease of the absorbance at 432 mμ (see Fig. 2) is considered to be highly significant. The analogously computed estimates of k' (rate of appearance of C'' as an asymptotic value at 245 mμ) and those determined graphically from the pseudo first order decrease of the 245 mμ maximum are also highly comparable.

It is concluded that the mechanism proposed in Eq. 6 is consistent with the experimental data. The analogously computed estimates of the absorptivities a, a', and a'' are also given in Table II. The a and a'' values are constant with change in pH, indicative of the absence of any functional groups affected by pH, and interacting with the chromophores. The a' values are not constant, indicative of the converse situation. They range from a value of 80,000 at pH 1.0 to 44,000 at pH values in excess of 6. Thus the a'_1 absorptivity may be assigned to the former numerical value and a_2' to the latter. The pKa of the C' functional group may be estimated by Eq. 14 as *ca.* 3.5. This value is consistent with the pKa previously estimated from the reversible 245 mμ absorbance changes of the solution of the C' intermediate.

Applications of Results of Kinetic Studies.—The results of these studies have great pharmaceutical utility. Below a pH of 7, the absorbance at the 432 mμ chromophore can be used as a valid estimate for the biological potency of streptovaricin, $[S]$, by the expression

$$[S] = \frac{A - a'[S]_o}{A - a'} = \frac{A - 2{,}500[S]_o}{12{,}500} \quad \text{(Eq. 15)}$$

where $[S]_o$ is the initial concentration in grams/liter and A is the absorbance at 432 mμ. Since the absorbance of the 432 mμ chromophore of streptovaricin C can be so correlated with the loss in biological activity below a pH of 9, the kinetic studies of the former can be used to calculate streptovaricin C stability at any pH ($-\log [H^+]$) below 9 at 30.3°. The appropriate equation is

$$\log \text{ (fraction of potency remaining)} = -kt/2.303 \quad \text{(Eq. 16)}$$

where k in 25% ethanol is given by Eq. 4.

Estimates of retention of potency in the stomach are possible. If pH values are assumed for the stomach, the half-life of streptovaricin C in the stomach is less than six hours for pH 1.0 and less than forty-

TABLE II.—APPARENT FIRST ORDER RATE CONSTANTS (IN SEC.$^{-1}$) AND ESTIMATES OF ABSORPTIVITY AT 244.5 mμ FOR THE DEGRADATION OF STREPTOVARICIN C $\xrightarrow{k}$ C' $\xrightarrow{k'}$ C''

pH	Analog Computer Estimates of Absorptivity $10^{-3}\,a''$	$10^{-3}\,a'$	$10^{-3}\,a$	Analog Computer Estimates 10^3k	$10^3k'$	First Order Plots 10^3k	$10^3k'$
1.12	46	80	25	160	25	260	18
1.97	46	74	26.5	53	12.5	48	9.8
2.73	47	62	27	16	3.7	9.8	3.2
4.38	46	48	26	27	..	10.5	3.9
5.41	45	45	26	17	..	13	5.5
6.22	46	44	26	17	..	12	5.7
7.42	46	39	29	24	..	15	7.4
8.57	46	44	26	17	..	17	6.5

[a] Absorptivity of C. [b] Absorptivity of C'. [c] Absorptivity of C''. [d] Based on first-order plots of absorbance disappearance at 432 mμ. [e] Based on first-order approach to final asymptote at 244.5 mμ.

three hours for pH 2. The effect of alcohol content, in general, is to decrease hydrolysis rates as is shown in the shift of k in sec.$^{-1}$ from 12×10^{-7} to 4.5×10^{-7} for an increase in alcohol content from 25% to 44% (see Table I). It may be concluded that significant loss of streptovaricin potency will occur in gastric contents below pH 1.5 if the stomach emptying time is assumed to be four hours.

It is obviously mandatory to refrigerate or freeze standard solutions and to extract or assay solutions in minimum time since the half-life of streptovaricin C should not be considered as more than fifty-three hours as calculated at 30° in 25% ethanol. However, standard solutions of high stability could be made in absolute alcohol.

A maximum yield of a hydrolytic intermediate, C', characterized by the 245 mμ maximum absorbance could be obtained in twenty-four hours in 0.1 M HCl, 25% ethanol at 30°.

An assay for streptovaricin C could be developed by permitting the streptovaricin complex of A, B, C, and D of concentration $[S]$ to stand in 25% ethanol at pH 8.5 for eight hours at 30°. The final bioassay should be largely streptovaricin C of concentration $[C]$. The original streptovaricin C concentration, $[C]_o$, of the solution could be calculated from

$$\log [C]_o = \log [C] + kt/2.303 \quad \text{(Eq. 17)}$$

where t is in seconds and k is 15×10^{-7} sec.$^{-1}$ The fraction, f, of C in the original complex could be estimated from

$$f = [C]_o/[S] \quad \text{(Eq. 18)}$$

This unique difference in the maintenance of bioactivity in aqueous solution provides an excellent rationale for the fact that C is the observed major component in the streptovaricin complex (8).

Thus, although the streptovaricins retain their structural anonymity, the observations on their physicochemical transformations permit the prediction of their routes and how to restrict their rates of travel.

SUMMARY

1. Streptovaricin C is degraded by specific hydrogen ion catalyzed and "spontaneous" hydrolysis. The degradation was followed by the loss of absorbance at 432 mμ and the appearance of a maximum absorbance at 245 mμ. The former in solutions below pH 9 is correlated with the loss in biological activity and can be used to calculate the nonhydrolyzed antibiotic remaining. Equations and constants are provided to calculate stability under various pH conditions.

2. General acid-base catalysis and oxidative degradation in solution were not observed. Increased alcohol content stabilized the streptovaricins; high stability results in absolute ethanol.

3. Streptovaricin C degrades to a biologically inactive intermediate C' characterized by enhanced absorbance at 245 mμ in acid. This absorbance is reversibly lost on neutralization indicative of a pKa *ca.* 3.5–4.0. An additional pKa *ca.* 10 was also indicated. This C' is further hydrolytically degraded with a loss of absorbance at all wavelengths to C'' and rate constants for this transformation have also been determined. A maximum yield of C' should be obtained in twenty-four hours in 0.1 M HCl and 25 per cent ethanol at 30°.

4. An assay for streptovaricin C could be developed by permitting the streptovaricin complex of A, B, C, and D to stand in 25 per cent ethanol for eight hours at pH 8.5 and at 30°. The final bioassay should be largely C. The original C concentration could be calculated from the data given.

5. In 0.01 N NaOH at 30°, the half-life of the streptovaricins is less than five minutes. The mechanism of alkaline degradation appears to differ from that of acid and "spontaneous" hydrolyses.

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The Solubility of Various Sulfonamides Employed in Urinary Tract Infections*

By F. J. BANDELIN† and WALTER MALESH

The solubilities of sulfonamides commonly employed in the therapy of urinary tract infections and their N^4 acetyl derivatives have been investigated in phosphate buffers and in a synthetic urine known as Mosher's Vehicle over the pH range 4.5 to 7.0 at 37°. All compounds increased in solubility with increasing pH and all had similar curve characteristics. Of the compounds investigated, sulfacetamide exhibited the greatest solubility, followed in decreasing order by sulfaethylthiadiazole, sulfamethylthiadiazole, sulfisoxazole, and the trisulfapyrimidines. The solubility characteristics of sulfamethoxypyridazine were found to differ from the other compounds of this group. The N^4 acetyl derivatives exhibited solubility curves similar to those of the parent compound but were generally less soluble in the media employed, with the exception of the combined trisulfapyrimidine acetyl derivatives. As a general rule, both the sulfonamides and their acetyl derivatives were more soluble in Mosher's Vehicle than in the phosphate buffers. Comparison of solubility data is made and details of the experimental methods given.

THE INSOLUBILITY of early sulfonamides and their acetyl derivatives in water and in tissue fluids, and the hazards of sulfonamide urolithiasis caused by the lack of solubility of these compounds had led to the development and introduction of sulfonamide compounds and mixtures of greater solubility and less danger of crystalluria.

Lehr (1) in 1945 introduced the mixed sulfonamides and with subsequent work (2–6) illustrated the value of the principle of separate solubilities for increasing the solubility of sulfonamides by such mixtures. This work was corroborated by Frisk and associates (7, 8) and by Oettingen and Cronheim (9) and led to the acceptance, in 1949, by the Council on Pharmacy and Chemistry of the American Medical Association of preparations containing two (10) or three (11) sulfonamides.

Soluble sulfonamides such as sulfacetamide, sulfamethylthiadiazole, and sulfisoxazole have become widely used as single entities and in combinations.

The essentiality of solubility for sulfonamides recommended for urinary infection has been stressed by a number of clinicians. Seneca, Henderson, and Harvey state (12), "In making a choice of sulfonamides for the treatment of urinary tract infections, a highly active, absorbable, and soluble compound which is excreted in high concentration in the urine is obviously sought." O'Connor (13) also states, "Sulfonamides of improved urinary solubility offer the cheapest and most easily tolerated drugs for routine office prescription."

Hawking and Lawrence state (14), "In determining the relative value of the sulfonamide compounds in urinary infections, numerous factors are concerned. The effectiveness depends upon the relative potency of the compound, on its concentration in the urine determined by the rate of excretion, solubility, on the degree of acetylation in the urine, and on the concentration in the kidney, ureter, and bladder wall."

Marshall (15), Yow (16), Tice (17), and Bourque and Joyal (18), among others, agree on the desirability of compounds of high solubility.

That high blood levels are not necessary to obtain a bactericidal urinary concentration with sulfonamides has been pointed out by Herrold (19), Cook (20), and others.

Since sulfonamides generally have a greater antibacterial effect against Gram-positive cocci than against Gram-negative bacilli such as *E. coli*, *Proteus*, *A. aeruginosa*, and *Ps. aeruginosa*, and since it is these latter which are largely responsible for urinary tract infections, it follows that higher concentrations of sulfonamide are required to inhibit these organisms.

In consideration of these various factors we investigated and determined the solubilities of sulfonamides generally recommended for urinary tract infections and their acetyl derivatives, both in phosphate buffer and in a synthetic urine, over the usual pH range of the urine (pH 4.5 to 7.0) and have plotted curves and derived values for comparison of solubilities at increasing pH increments as hereinafter described.

EXPERIMENTAL

Preparation of Sulfonamide and Acetyl Sulfonamide Solutions in Buffers.—The buffer systems employed in this work were essentially the phosphate buffers of Gortner (21) which were prepared at four times the concentrations given by the

* Received June 21, 1958, from the Research Laboratories, Flint, Eaton and Co., Decatur, Ill.
† Present address: Strong, Cobb & Co., Inc., Cleveland 4, Ohio.

author.[1] The two solutions were mixed to obtain buffer solutions of precise pH values, increasing in 0.5 pH increments from 4.5 to 8.0 as required. The pH values of all buffer mixtures were determined with a pH electrometer and adjustments made, where necessary, to produce buffers of accurate values.

Solutions were prepared by adding an excess of the sulfonamide or its acetyl derivative to 10 ml. of buffer solution at each pH level in 18 x 150-mm. test tubes, stoppering the tubes, placing in a water bath at 37° with gentle agitation for twenty-four hours to allow equilibrium to be reached. At the end of this time, the mixture was filtered through a dry E and D No. 192 fluted filter paper, and a 1-ml. aliquot was accurately pipetted into a volumetric flask for dilution and analysis. The balance was retained for pH determination to ascertain any change in pH value. This latter value was designated as the equilibrium pH. All solutions were prepared in duplicate, and duplicate samples were used for analysis throughout.

Preparation of Sulfonamide and Acetyl Sulfonamide Solutions in Synthetic Urine.—The synthetic urine employed, known as the Mosher Vehicle (22), is made up of the following per liter of water: sodium chloride 2.950 Gm., potassium chloride 1.660 Gm., magnesium chloride 0.121 Gm., calcium chloride 0.143 Gm., ammonium phosphate 0.300 Gm., and urea 20 Gm. To 10-ml. aliquots of this solution in 18 x 150-mm. test tubes at 37°, an excess of the sulfonamide or its acetyl derivative was added followed by sufficient one per cent H_3PO_4 or one per cent NaOH to adjust to the required pH value, thus producing solutions increasing by 0.5 pH increment from 4.5 to 7.0. The solutions were agitated in a 37° water bath for twenty-four hours. Meanwhile acid or base was added to keep the solutions at the desired pH level until equilibrium in pH and in concentration was attained. Since the Mosher Vehicle has little buffer capacity it was found necessary to adjust these solutions to the exact pH points of investigation otherwise the shift in pH due to the solubility of some of the sulfonamides was so great as to throw the final pH value outside of the range of investigation. After equilibrium for twenty-four hours at 37°, the solution was filtered as above and an aliquot pipetted into a volumetric flask for analysis.[2]

Sulfonamide Determination.—The colorimetric method employed for the determination of the sulfonamide content of the solutions was essentially that of Bratton and Marshall as described in detail by Biamonte and Schneller (23). Standard curves were prepared for the individual sulfonamides using accurately prepared standard solutions.

All solutions of the acetyl derivatives were hydrolyzed by refluxing with 5% sulfuric acid for one hour to liberate the free amino compound before determination.

Determinations were made by reading the absorbance of the solution, after color development, at 545 mμ with a spectrophotometer using a reagent blank. Values obtained were read from the standard curves with appropriate factoring for the dilution. All solubilities are stated in mg. per 100 ml. of solution.

All determinations were run in duplicate and the average values at resultant pH values are given in Table I. Values for the acetyl derivatives are plotted as free sulfonamides.

Comparative solubilities of sulfonamides at 0.5 pH unit increments over the pH range 4.5 to 7.0 in phosphate buffers at 37° as obtained by extrapolation of the solubility curves derived from data are given in Table II. The same information on the acetyl derivatives is given in Table III. Figure 1 is included to illustrate graphically the comparative solubilities given in Table II.

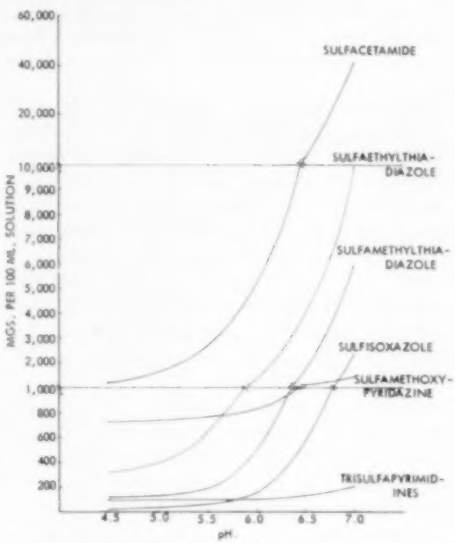

Fig. 1.—Solubilities of various sulfonamides in phosphate buffers at 37°.

DISCUSSION

As is apparent from the data presented, the differences in the solubilities of these various sulfonamides in the media employed, is considerable. The acetyl derivatives show similar differences in relative solubility, being less soluble than the parent compound with the exception of the trisulfapyrimidines, in which case the reverse is true as has already been demonstrated (23). As a general rule, both the sulfonamides and their acetyl derivatives were slightly more soluble in Mosher's Vehicle than in the phosphate buffers. This appears to be due to a direct solubilizing action of the urea upon the sulfonamide compounds.

While the sulfonamides have generally been considered as poorly soluble compounds, several of these compounds show marked solubility, especially at the higher pH values. Sulfacetamide, for instance, is soluble in excess of 40% at pH 7.0, while at this pH the remainder of the compounds had the following solubilities: sulfamethylthiadiazole 5.96%, sulfi-

[1] Buffers consisted of varying mixtures of the following solutions: dibasic sodium phosphate, heptahydrate—71.6 Gm. per L. of distilled water; monobasic potassium phosphate—36.3 Gm. per L. of distilled water.

[2] At pH 7.0 some precipitation of calcium and magnesium hydroxides occurred in the synthetic urine. This was filtered off with the excess sulfonamide.

TABLE I.—SOLUBILITY OF VARIOUS SULFONAMIDES AND THEIR ACETYL DERIVATIVES IN PHOSPHATE BUFFERS AND IN SYNTHETIC URINE OVER THE pH RANGE 4.5 TO 7.0

Milligrams of Compound Soluble in 100 ml. of Buffer at 37°				Milligrams of Compound Soluble in 100 ml. of Synthetic Urine at 37°			
Initial pH	Triple Sulfonamide	Equilibrium pH	Acetyl Triple Sulfonamide	Equilibrium pH	Triple Sulfonamide	Acetyl Triple Sulfonamide	Equilibrium pH
4.5	96	4.5	116	4.5	100	218	4.5
5.0	98	5.0	121	5.0	108	223	5.0
5.5	102	5.5	132	5.5	118	231	5.5
6.0	109	6.0	158	6.0	136	254	6.0
6.5	130	6.4	216	6.4	182	163	6.5
7.5	209	7.1	490	7.1	275	630	7.0
	Sulfacetamide		Acetyl Sulfacetamide		Sulfacetamide	Acetyl Sulfacetamide	
4.5	..	..	60	4.5	1,500	240	4.5
5.0	1,250	4.5	125	5.0	1,950	310	5.0
5.5	1,350	4.8	250	5.5	3,150	505	5.5
6.0	2,150	5.3	550	5.8	6,000	1,050	6.0
6.5	3,020	5.6	1,150	6.2	15,000	2,520	6.5
7.0	4,400	5.9	2,310	6.6	50,000	5,600	7.0
7.5	15,000	6.6	3,900	7.0			
8.0	41,000	7.0	3,900	7.0			
	Sulfamethyl-thiadiazole		Acetyl Sulfamethyl-thiadiazole		Sulfamethyl-thiadiazole	Acetyl Sulfamethyl-thiadiazole	
4.5	105	4.5	41	4.5	120	10	4.5
5.0	125	5.0	50	5.0	150	21	5.0
5.5	200	5.5	71	5.5	260	45	5.5
6.0	470	6.0	102	6.0	620	145	6.0
6.5	1,000	6.3	260	6.5	1,980	380	6.5
7.0	1,990	6.6	630	6.9	8,400	995	7.0
8.0	9,250	7.3	2,400	7.3			
	Sulfisoxazole		Acetyl Sulfisoxazole		Sulfisoxazole	Acetyl Sulfisoxazole	
4.5	33	4.5	8	4.5	36	30	4.5
5.0	45	5.0	12	5.0	51	44	5.0
5.5	70	5.5	38	5.5	80	70	5.5
6.0	175	6.0	105	6.0	220	160	6.0
6.5	405	6.4	190	6.4	710	560	6.5
7.0	1,360	6.8	375	6.7	2,600	1,230	7.0
7.5	2,870	7.2	1,040	7.1			
	Sulfaethyl-thiadiazole		Acetyl Sulfaethyl-thiadiazole		Sulfaethyl-thiadiazole	Acetyl Sulfaethyl-thiadiazole	
4.5	..	..	140	4.4	360	225	4.5
5.0	325	4.6	162	4.7	380	230	5.0
5.5	465	5.2	212	5.2	440	250	5.5
6.0	760	5.6	300	5.6	480	350	6.0
6.5	2,250	6.2	510	6.35	600	650	6.5
7.0	5,900	6.6	740	6.7	1,875	1,140	7.0
7.5	7,300	6.8	1,175	7.1			
8.0	17,000	7.1					
	Sulfamethoxy-pyridazine		Acetyl Sulfamethoxy-pyridazine		Sulfamethoxy-pyridazine	Acetyl Sulfamethoxy-pyridazine	
4.5	720	4.5	22	4.5	460	165	4.5
5.0	740	4.8	24	5.0	466	168	5.0
5.5	770	5.4	26	5.5	475	174	5.5
6.0	800	5.9	28	6.0	488	182	6.0
6.5	920	6.2	30	6.5	552	212	6.5
7.0	1,380	7.0	41	7.0	862	290	7.0

TABLE II.—COMPARATIVE SOLUBILITIES AT 0.5 pH UNIT INCREMENTS OVER THE RANGE 4.5 TO 7.0 IN PHOSPHATE BUFFERS AT 37°

	Mg. per 100 ml. of Solution					
	4.5	5.0	5.5	6.0	6.5	7.0
Trisulfapyrimidines	96	98	102	109	139	192
Sulfacetamide	1,250	1,590	2,760	5,400	11,300	41,000
Sulfamethylthiadiazole	105	125	200	470	1,820	5,960
Sulfisoxazole	33	45	70	175	510	2,350
Sulfaethylthiadiazole	320	450	660	1,520	4,050	10,800
Sulfamethoxypyridazine	720	740	780	840	1,140	1,380

TABLE III.—COMPARATIVE SOLUBILITIES OF ACETYL SULFONAMIDES AT 0.5 pH UNIT INCREMENTS OVER THE RANGE 4.5 TO 7.0 IN PHOSPHATE BUFFERS AT 37°

Acetyl Derivative	Mg. per 100 ml. of Solution					
	4.5	5.0	5.5	6.0	6.5	7.0
Trisulfapyrimidines	116	121	132	158	230	420
Sulfacetamide	60	125	250	790	1,980	3,900
Sulfamethylthiadiazole	41	50	71	102	260	790
Sulfisoxazole	8	12	38	105	220	710
Sulfaethylthiadiazole	150	190	280	310	620	1,040
Sulfamethoxypyridazine	22	24	26	28	30	41

soxazole 2.35%, sulfamethoxypyridazine 1.38%, and trisulfapyrimidines 0.19%. Sulfaethylthiadiazole exhibited a much higher solubility in phosphate buffer than it did in Mosher's Vehicle, being soluble to the extent of 10.70% in the former at pH 7.0.

The points on the pH scale at which marked acceleration of solubility occurred also varied. In the case of the trisulfapyrimidines a marked increase in solubility commenced at around pH 6.0; with sulfisoxazole, sulfamethylthiadiazole, and sulfacetamide this was evidenced at about pH 5.0 and above and with sulfaethylthiadiazole solubility increased abruptly at pH 5.5 and above. The solubility of sulfamethoxypyridazine remained rather constant over the pH range to about pH 6.0 after which it rose precipitously.

Although the compounds differed markedly in solubility, all curves were characteristically similar with the exception of sulfamethoxypyridazine. This compound was unique in showing somewhat different curve characteristics and in the fact that it was found to be considerably more soluble in phosphate buffer than in Mosher's Vehicle. The acetyl derivative of this compound, however, was similar to the acetyl derivatives of the other compounds in that it was considerably more soluble in Mosher's Vehicle than in the phosphate buffers.

Although the urine of normal individuals is usually considered to have a mean pH of 6.0, it may range from 4.8 to 7.5 (24) in pathological conditions. This pH range is therefore critical in view of the facts here illustrated showing that the solubility of most of the compounds investigated increased 50 to 100-fold from pH 5.0 to pH 7.0. With the trisulfapyrimidines the increase is only three-fold and its combined solubilities are the lowest of the entire group.

Since urinary acidity is increased in conditions where the urine is concentrated, as in fevers (25), solubility is important because as the urine is concentrated, so also is its sulfonamide content, and, as the urinary acidity increases with concentration, the solubility of sulfonamide is markedly decreased, thus producing the possibility of sulfonamide urolithiasis. If normal fluid volume and pH are maintained, it is doubtful if any of the above sulfonamides, with their adequate solubilities, would be dangerous. However, sulfisoxazole and sulfamethoxypyridazine have rather insoluble acetyl derivatives, and it may be that when present in high concentration in acid urine, urolithiasis may be a factor. This would be dependent, however, upon the dose, the rate of excretion, and the extent of acetylation.

SUMMARY

The solubilities of six sulfonamides commonly employed in urinary tract infections and their acetyl derivatives have been investigated over the pH range 4.5 to 7.0 at 37° in phosphate buffers and in a synthetic urine known as Mosher's Vehicle. Of these compounds, sulfacetamide was the most soluble followed, in decreasing order, by sulfaethylthiadiazole, sulfamethylthiadiazole, sulfisoxazole, and trisulfapyrimidines. Sulfamethoxypyridazine was intermediate in the group in that it was not so much affected by pH and was relatively more soluble in the acid region than any of the other compounds with the exception of sulfacetamide. Of the acetyl derivatives, sulfacetamide was also the most soluble at the higher pH levels while at a pH of 5.5 and below, sulfaethylthiadiazole was the more soluble. The combined acetyl derivatives of the trisulfapyrimidines were somewhat more soluble below pH 6.0 than was the acetyl sulfamethylthiadiazole. The latter exceeded the solubility of the former at pH 6.5 and above. Acetyl sulfisoxazole exhibited a rather low solubility at pH levels under 6.5 but increased greatly at higher pH values. Acetyl sulfamethoxypyridazine had a very low solubility over the entire pH range in the buffers.

All of the sulfonamides were somewhat more soluble in Mosher's Vehicle than in the buffers with the exception of sulfamethoxypyridazine which was somewhat more soluble in the phosphate buffers. At pH 6.0 (mean pH of urine) acetyl sulfacetamide was the most soluble of the acetyl derivatives followed by the acetyl derivatives of sulfamethylthiadiazole, sulfamethoxypyridazine, sulfaethylthiadiazole, sulfisoxazole, and trisulfapyrimidines. The acetyl derivatives were also more soluble in Mosher's Vehicle than in the buffer with acetyl sulfamethoxypyridazine being considerably more soluble in Mosher's Vehicle than in the buffer.

The physiological implications of these observations are briefly discussed.

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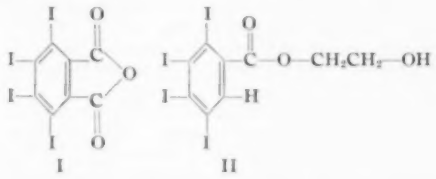
New Iodine Compounds for Hepatolienography*

By ROBERT J. DUMMEL, JAMES D. MADDEN, and SYDNEY F. THOMAS

A new source of radio-opaque compounds for the development of hepatolienography contrast media consists of the class of tetraiodophthalic anhydride derivatives. Hydroxyethyl and hydroxypropyl tetraiodobenzoates were prepared by the decarboxylation of hydroxyethyl and hydroxypropyl tetraiodophthalates. These compounds gave good colloidal dispersions by precipitation in water from acetone. Zirconyl tetraiodophthalate was prepared as a colloidal precipitate, but hydrolysis took place in attempts to neutralize the solvent.

A PERSISTENT SEARCH has been under way to replace thorium dioxide (Thorotrast) as an intravenous colloidal contrast media for the opacification of the liver and spleen, because of the long-term damage to the sites of deposition caused by the permanent radioactive deposits (1). Experiments with tantalum oxide, tungsten oxide (2), zirconium oxide (3), and stannic oxide (4) have been reported. Elimination of the contrast media from the sites of deposition after completion of the diagnosis is desirable. Metal oxides are permanently fixed, but organic iodine compounds offer a possible alternative, if one can be found which will undergo slow metabolic breakdown. Experiments with emulsions of iodinated vegetable oils (Lipiodol) and ethyl esters of iodinated fatty acids (Angiopac and Ethiodol) have been reported (5, 6, 7).

An ideal compound for hepatolienography should be chemically inert, water insoluble, easily dispersed to a stable colloid, and subject to slow elimination from the organism. Among the organic iodine compounds, nearly all soluble injectable contrast media used today are aromatic in structure. We therefore chose as a starting material for a series of possible contrast media the aromatic compound tetraiodophthalic anhydride (I), which contains by weight 77.9 per cent iodine.



I II

The chemically reactive anhydride group can give rise to an open ended series of derivatives such as monoesters, diesters, amides, and imides. Such derivatives should be hydrolyzed metabolically. Information on the chemistry of tetraiodophthalic anhydride was limited to accounts of its synthesis (8), and the formation of the monomethyl and dimethyl esters (9), dioctadecenyl and didodecyl esters (10), several aliphatic imides (11), the anil and phthalein (12).

The results reported below were encountered in attempts to prepare a polyester from ethylene glycol and tetraiodophthalic anhydride by a reaction analogous to the well-known preparation of polymer resins from glycols and phthalic anhydride. Tetraiodophthalic anhydride reacted readily with hot ethylene glycol to give the half-ester, hydroxyethyl tetraiodophthalate. This crystalline solid reverted on dry heating to the anhydride and the glycol. When the half-ester was refluxed in a high-boiling solvent, a resinous, low-melting solid was obtained, which was separated into a crystalline solid and an amorphous resin. The crystalline solid was identified as hydroxyethyl tetraiodobenzoate (II),

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We wish to acknowledge the support of this research by grants from the California Division of the American Cancer Society and the Atomic Energy Commission.

which resulted from the thermal decarboxylation of the half-ester. Precedent for this reaction was found in the reported reactions of tetrachlorophthalic anhydride (13). The presence of polyester in the uncrystallized resin was inferred from hydrolysis to tetraiodophthalic acid, but final proof must depend on molecular weight studies.

The same procedure using tetraiodophthalic anhydride with propylene glycol gave similar products. A crystalline product was identified as hydroxypropyl tetraiodobenzoate.

The low reported toxicity of zirconyl salts (3) and tetraiodophthalate (14) were the basis of attempts to prepare zirconyl tetraiodophthalate.

EXPERIMENTAL

Tetraiodophthalic Anhydride.—The commercially available (Eastman Kodak) product, m. p. 310–322°, was used without further purification. It was found to be readily soluble in dioxane and dimethylformamide, from which recrystallization could be achieved by the addition of chloroform, cyclohexane, or acetic acid. Heating tetraiodophthalic anhydride, alone or in a solvent, above 350° resulted in decomposition with liberation of iodine. Sodium, potassium, and ammonium mono-, and di-salts were prepared by the addition of the calculated amount of base to a dioxane solution of tetraiodophthalic anhydride.

Anal.—A sample was fused with NaOH in a nickel crucible, neutralized, and titrated with standard silver nitrate according to the Volhard method, using tetraiodofluorescein for an indicator. Calcd. for $C_8I_4O_3$: I, 77.88. Found: I, 77.77.

Hydroxyethyl, Hydroxypropyl, and Ethyl Tetraiodophthalates.—Half-esters were readily formed by dissolving the anhydride in refluxing alcohols. Samples of the anhydride were dissolved in ethylene glycol, propylene glycol, and for comparison, in ethanol. In each case, a crystalline yellow solid was obtained. Small samples of each product were heated in a capillary melting point apparatus, with evolution of vapor, and a residue which melted at 205–222°. The melting point was not depressed by mixing the sample with tetraiodophthalic anhydride.

Equivalent Weights.—Calcd. for hydroxyethyl tetraiodophthalate, $C_{10}H_6I_4O_5$: 714. Found: 710. Calcd. for hydroxypropyl tetraiodophthalate, $C_{11}H_8I_4O_5$: 728. Found: 726. Calcd. for ethyl tetraiodophthalate, $C_{10}H_6I_4O_5$: 714. Found: 710.

Hydroxyethyl Tetraiodobenzoate.—Hydroxyethyl tetraiodophthalate (10.8 Gm.), or the equivalent mixture of ethylene glycol and tetraiodophthalic anhydride, was refluxed four hours in dimethylformamide (50 ml.). Evaporation of the solvent under reduced pressure left a dark, opaque residue (17 Gm.). The residue was refluxed with acetone (100 ml.), and the acetone solution was filtered and evaporated to a cloudy amber resin (9.9 Gm.). The cloudy amber resin was refluxed in dichloromethane (100 ml.), and the solution filtered from an amorphous yellow powder. Evaporation of the solvent left a transparent glassy amber resin (8.6 Gm.) which softened to a viscous oil at 90–100°, containing 75.02% iodine. The apparent equivalent weight of 5,520 indicated some unreacted acid. Part of this resin was dissolved in dichloromethane (100 ml.) and extracted with 10% sodium carbonate solution (2 × 100 ml.). The organic phase, considerably lighter in color, was washed with water, dried over calcium chloride, filtered and evaporated to a light yellow oil (4.7 Gm.). Recrystallization from acetone gave a light yellow, semicrystalline solid, m. p. 215–224°.

Anal.—Calcd. for $C_9H_6I_4O_3$: I, 75.59. Found: I, 75.44.

Hydroxypropyl Tetraiodobenzoate.—Starting with hydroxypropyl tetraiodophthalate, or the equivalent mixture of propylene glycol and tetraiodophthalic anhydride, the same procedure as above was repeated to give a similar product. The product was light yellow semicrystalline solid, m. p. 215–222°, and soluble in acetone and dichloromethane.

Anal.—Calcd. for $C_{10}H_8I_4O_3$: I, 74.22. Found: I, 74.32.

Zirconyl Tetraiodophthalate.—The addition of zirconyl chloride (50.2 Gm., 0.153 mole) in water (500 ml.) to a solution of disodium tetraiodophthalate (106 Gm., 0.149 mole) in 1:3 dioxane:water (1,000 ml.) precipitated a pale yellow colloidal dispersion. The precipitate was washed by centrifugation and resuspension, and dried to a pale yellow powder (63 Gm., 0.812 mole, 55% yield).

Anal.—Calcd. for $C_8I_4O_3Zr$: ZrO_2, 15.9. Found: ZrO_2, 16.7. The initial precipitate was stable only in acid solution, and attempts to neutralize the solution with dilute base resulted in hydrolysis to zirconium hydroxide and sodium hydrogen tetraiodophthalate.

Preliminary Dispersion Studies. Hydroxyethyl and hydroxypropyl tetraiodobenzoate were each dissolved in acetone, and the acetone solutions poured into water. In each case, a colloidal dispersion formed with very little gummy precipitation. The acetone was removed and the dispersions concentrated by distillation under reduced pressure. Microscopic examination of these dispersions revealed uniformly spherical particles in rapid Brownian motion, with the estimated average particle size of 1 μ. The dispersions settled slowly during several days, and could be partly resuspended by shaking. The half-esters in this series did not give stable dispersions by this technique.

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Metal Chelates and Antitubercular Activity V*

Riboflavin, Riboflavin-5′-phosphate, Alloxan, Phthaloylhydrazine

By WILLIAM O. FOYE, FRED B. BLOCK, and WINTHROP E. LANGE

Metal complexes, which are probably chelates, have been prepared of riboflavin, roboflavin-5′-phosphate, alloxan, and phthaloylhydrazine using divalent copper, cobalt, nickel, iron, and zinc ions. Antitubercular tests in mice showed these chelates to be essentially inactive.

PREVIOUS ATTEMPTS to find a relationship between metal-chelating ability and antituberculotic activity have shown some success. A number of agents which have been employed against human tuberculosis have shown an ability to chelate heavy metal ions (1, 2, 3) and several have been shown to retain activity in the form of heavy metal chelates (4, 5, 6). An example of a class of agents which showed a limited amount of activity in the mouse in the chelated form while the nonchelated agents were inactive has also been found in the case of the *o*-hydroxyazonaphthols (7). In order to inquire further into the possibility that the action of metal chelates against tuberculosis is of fairly general occurrence or whether it is dependent upon limited structural requirements, several other types of metal chelates have been prepared and tested in the mouse. For this purpose, chelates of riboflavin, riboflavin-5′-phosphate, alloxan, and phthaloylhydrazine were used.

METHODS OF PREPARATION

The preparation of metal chelates of riboflavin (8) and of alloxan (9) has already been reported. Chelation of riboflavin-5′-phosphate with nickel and cobaltous chlorides was first carried out at pH 9 using the procedure of Foye and Lange (8) for riboflavin chelates. There was a distinct drop in pH when the alkalinity of the reaction was not maintained, and the isolation of colored, water-insoluble products provided further indication that chelation was taking place. In addition, negative tests for metal ions in the reaction mixture were obtained with ferrocyanide after the reactions were complete. These chelates were insoluble in organic solvents, water, and aqueous alkali and were decomposed by aqueous acid. Since recrystallization was impossible, excess riboflavin-5′-phosphate and sodium ion were removed by Soxhlet extraction using water. Elemental analysis of the dried products revealed an unusually high metal content (see Table II).

In an attempt to prepare chelates having a lower metal content, reactions with several metal ions were carried out at pH 7. Evidence for chelation was again noted by a drop in pH in experiments where the pH was not maintained by alkali. The following drops in pH were noted: with zinc ion, the pH dropped to 4.2, with iron to 5.2, with cobalt to 5.6, with nickel to 5.6, and with copper to 5.8. In the preparation done at pH 7, colored, water-insoluble products were again obtained. Very slight positive tests for metal ion were noted in the reaction mixtures, however, after the reactions were complete. Elemental analysis of these chelates showed in general a ratio of three metal ions to two molecules of riboflavin-5′-phosphate. This amount of metal in the products was still considered somewhat high for animal experimentation, however.

Aqueous mixtures of the products prepared at either pH 7 or pH 9 were then brought to pH 3 to reduce the metal contents still further. Both soluble and insoluble fractions were isolated from this treatment of the cobalt and nickel chelates, but the two fractions from the nickel chelate proved to be identical. The copper and zinc chelates gave only insoluble products, while the iron chelate was unaffected by this treatment. Products having a ratio of two riboflavin-5′-phosphate molecules to one metal ion were obtained from the cobalt, nickel, and zinc chelates, whereas the copper chelate was converted from a ratio of five metal ions to two molecules of riboflavin-5′-phosphate at pH 7 to a ratio of 3:2 at pH 3.

The analytical results of the various riboflavin-5′-phosphate chelates prepared are listed in Table II. The general analytical procedure consisted first of a metal analysis of the product dried *in vacuo* (2–5 mm.) at room temperature. Carbon-hydrogen analyses were then obtained on the products after being dried at a higher vacuum (1 mm.) and the loss of weight observed on this second drying generally corresponded to the theoretical amount for two or three molecules of water. This procedure provided a check on the quantity of water of coordination present, since the Karl Fischer or complete dehydration methods of water analysis failed to give consistent results with these compounds. The color and per cent yield of these products are recorded in Table I.

Chelation reactions of phthaloylhydrazine were carried out with divalent copper, cobalt, and nickel ions at a pH of 9. The insoluble products were purified by washing with water and extraction with acetone. A characterizable ferrous chelate was not obtained. The analytical results indicate a 1:1 ratio of metal ion to phthaloylhydrazine (Table III), but the actual structures probably have a 2:2 ratio. This is

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indicated by the absence of bound water in the copper derivative, where the coordination capacity of four would be satisfied by two molecules of phthaloylhydrazine, and the presence of only two molecules of water for each cobalt or nickel ion, where the usual coordination capacity is six.

BIOLOGICAL ACTIVITY

Antitubercular tests on nonchelated agents of the types under discussion have been reported only for phthaloylhydrazine. Buu-Hoï (10) found this compound to be active against avian tuberculosis in 1946. O- and N-Substituted derivatives of phthaloylhydrazine were later found to be markedly active against human tuberculosis in mouse tests, however (11).

Tests against experimental tuberculosis in mice were carried out at the Lilly Research Laboratories on a number of the compounds discussed above. The copper, cobalt, nickel, iron, zinc, and manganese chelates of riboflavin (8) were essentially inactive when tested at levels of 0.25, 0.1, and 0.02% in the diet. The iron chelate did show slight activity at 0.02%, however. Among the riboflavin-5′-phosphate chelates, the following were tested: the cupric derivative having a 2:3 ratio of ligand to metal, the zinc compound having a 2:1 ratio, the 2:1 cobalt derivative, and the 2:3 ferrous compound. Although these compounds were described as essentially inactive, small increases in survival time were noted at some dietary levels. These results are shown in Table IV.

The 1:1 cobalt chelate of alloxan (9) was tested at dietary levels of 0.01 and 0.05%, and it was found to be toxic at the higher level and inactive at the lower. The cobalt and nickel complexes of phthaloylhydrazine were tested at dietary levels of 0.01 and 0.5%. The cobalt derivative was toxic at the higher level and inactive at the lower, while the nickel derivative was inactive at both levels.

EXPERIMENTAL

Metal Chelates of Riboflavin-5′-phosphate at Various pH's.

pH 9.—Two grams (0.0039 mole) of sodium riboflavin-5′-phosphate (Nutritional Biochemicals Corp.) was added to 50 ml. of water in a beaker equipped with a mechanical stirrer. To the solution was added sufficient 5% sodium hydroxide solution to bring the pH to 9. An aqueous solution of 0.83 Gm. (0.0035 mole) of cobalt (II) chloride hexahydrate or nickel (II) chloride hexahydrate was then added slowly over a period of ten minutes, along with sufficient 5% alkali to maintain the pH at 9. After thirty minutes more of stirring and adjusting the pH, the mixture was centrifuged, and the residue was dried *in vacuo*. The supernatant liquid was made neutral with 5% hydrochloric acid, reduced in volume by evaporation, and treated with ethanol to precipitate excess riboflavin-5′-phosphate. The dried residue was extracted with water in a Soxhlet extractor for twenty-four hours and was then dried *in vacuo* (2–5 mm.) over phosphorus pentoxide for twelve to sixteen hours.

pH 7.—To 50 ml. of water in a beaker equipped with a mechanical stirrer was added 2.0 Gm. (0.0039 mole) of sodium riboflavin-5′-phosphate. To this solution was added sufficient 5% sodium hydroxide solution to bring the pH to 7. An aqueous solution of 0.0039 mole of cobalt (II) chloride hexahydrate, nickel (II) chloride hexahydrate, zinc (II) chloride, ferrous sulfate heptahydrate, or cupric chloride dihydrate was then added slowly over a period of ten minutes, along with sufficient 5% alkali to maintain the pH at 7. After thirty minutes more of stirring and adjusting the pH, the product was isolated and purified as in the preceding procedure.

pH 3.—To 25 ml. of water in a beaker equipped with a mechanical stirrer was added 1.0 Gm. of metal chelate prepared either at pH 7 or 9. The mixture was brought to a pH of 3 by the addition of 5% hydrochloric acid. After one hour of stirring and adjusting the pH, the mixture was filtered and the residue was washed with water and methanol. The product was dried *in vacuo* (2–5 mm.) over phosphorus pentoxide for twelve to sixteen hours.

To the filtrate was added three to four volumes of acetone to precipitate any water-soluble complex. Nearly complete evaporation of the filtrate generally gave the same result as the addition of acetone. The solid was collected, washed with methanol, and dried in the usual manner.

TABLE I.—METAL CHELATES OF RIBOFLAVIN-5′-PHOSPHATE

Formula[a]	Prepared, pH	Color[b]	Yield, %
$R\text{-}Co_6\cdot 13H_2O$	9	Brown	90
$R_2\text{-}Co_3\cdot 7H_2O$	7	Yellow-brown	100+
$R_2\text{-}Co\cdot 6H_2O$	3	Dark yellow	77
$R\text{-}Ni_6\cdot 17H_2O$	9	Gold	97
$R_2\text{-}Ni_3\cdot 7H_2O$	7	Bright orange	98
$R_2\text{-}Ni\cdot 4H_2O$	3	Gold-orange	81
$R_2\text{-}Zn_3\cdot 12H_2O$	7	Bright orange	89
$R_2\text{-}Zn\cdot 4H_2O$	3	Gold-orange	100+
$R_2\text{-}Fe_3\cdot 7H_2O$	7	Rust	90
$R_2\text{-}Fe_3\cdot 6H_2O$	3	Rust	99
$R_2\text{-}Cu_3\cdot 13H_2O$	7	Yellow-brown	89
$R_2\text{-}Cu_3\cdot 4H_2O$	3	Orange	89

[a] R=$C_{17}H_{21}N_4O_9P$ minus two hydrogen atoms for each metal ion in the formula.
[b] Sodium riboflavin-5′-phosphate (Nutritional Biochemicals Corp.) has a gold-orange color.

Metal Chelates of Phthaloylhydrazine

Phthaloylhydrazine (0.01 mole) prepared by the method of Barber and Wragg (12), was dissolved in 50 ml. of water and 5% sodium hydroxide solution to give a pH of 9. An aqueous solution of the metal chloride (copper (II), cobalt (II), or nickel (II)) was then added slowly with stirring, and the pH was maintained at 9. The reaction mixture was stirred for an hour and then allowed to settle overnight. The products were filtered, washed with water, and extracted with acetone in a Soxhlet extractor for three hours. They were dried in air at room temperature. A 72% yield of pale green nickel chelate, a 90% yield of maroon cobalt chelate, and an 82% yield of green copper chelate were obtained, based on the formulas in Table III. It was necessary to centrifuge the reaction mixture to obtain the copper derivative.

Metal Analyses

Cobalt.—A modification of the method of Hillebrand, *et al.* (13), was used. A dilute hydrochloric

TABLE II.—ANALYSES OF THE RIBOFLAVIN-5′-PHOSPHATE CHELATES

Formula[a]	Prepared, pH	Calculated, %				Found, %[b]			
		C	H	M^{++}	H_2O[c]	C	H	M^{++}	H_2O[d]
R-Co_6·$13H_2O$	9	..	..	34.56	5.24	..	..	35.54	5.94
R-Co_6·$10H_2O$		20.88	2.99	..	..	20.76	3.36	..	..
R_2-Co_3·$7H_2O$	7	..	..	14.62	5.96	..	..	14.54	5.93
R_2-Co_3·$3H_2O$		35.90	3.72	..	..	35.92	3.81	..	..
R_2-Co·$6H_2O$	3	..	..	5.47	3.34	..	..	5.03	3.20
R_2-Co·$4H_2O$		39.20	4.64	..	..	39.72	4.75	..	..
R-Ni_6·$17H_2O$	9	..	..	31.94	3.27	..	..	32.39	2.63
R-Ni_6·$15H_2O$		19.14	3.69	..	..	19.10	4.41	..	..
R_2-Ni_3·$7H_2O$	7	33.78	4.17	14.57	..	34.31	4.68	13.04	..
R_2-Ni·$4H_2O$	3	..	..	5.64	3.46	..	..	5.52	3.08
R_2-Ni·$2H_2O$		40.61	4.41	..	..	40.37	4.83	..	..
R_2-Zn_3·$12H_2O$	7	..	..	14.87	2.73	..	..	13.99	2.92
R_2-Zn_3·$10H_2O$		31.83	4.40	..	..	31.65	3.49	..	..
R_2-Zn·$4H_2O$	3	..	..	6.24	3.44	..	..	9.02	4.26
R_2-Zn·$2H_2O$		40.35	4.38	..	..	40.16	4.23	..	..
R_2-Fe_3·$7H_2O$	7	..	..	13.96	9.01	..	..	12.84	8.46
R_2-Fe_3·H_2O		37.39	3.51	..	..	37.68	4.12	..	..
R_2-Fe_3·$6H_2O$	3	..	..	14.17	4.57	..	..	12.35	4.11
R_2-Fe_3·$3H_2O$		36.19	3.75	..	..	36.54	4.08	..	..
R_2-Cu_3·$14H_2O$	7	27.73	4.11	21.57	..	28.06	3.94	21.69	..
R_2-Cu_3·$6H_2O$	3	..	..	15.81	2.99	..	..	14.60	3.28
R_2-Cu_3·$4H_2O$		34.92	3.79	..	..	35.26	4.07	..	..

[a] R=$C_{17}H_{21}N_4O_9P$ minus two hydrogen atoms for each metal ion in the formula.
[b] Carbon and hydrogen analyses were done by the Clark Microanalytical Laboratory, Urbana, Illinois and the Weiler and Strauss Microanalytical Laboratory, Oxford, England. Samples were dried at room temperature *in vacuo* (1 mm.).
[c] This quantity represents the difference in theoretical water content shown by the two consecutive formulas listed under a given pH (e. g., $3H_2O$ for R—Co_6·$13H_2O$ → R—Co_6·$10H_2O$).
[d] This quantity represents the loss of weight on drying at room temperature at 1 mm. pressure the product previously dried at room temperature at 2–5 mm. pressure.

TABLE III.—ANALYSES OF THE PHTHALOYLHYDRAZINE CHELATES

Formula	Calculated, %				Found, %[a]			
	C	H	M^{++}	H_2O	C	H	M^{++}	H_2O[b]
$C_{16}H_8N_4O_4Co_2$·$4H_2O$	37.67	3.16	22.94	15.2	38.24	3.22	22.71	14.9
$C_{16}H_8N_4O_4Ni_2$·$4H_2O$	37.69	3.18	..	15.2	37.41	3.33	..	16.9
$C_{16}H_8N_4O_4Cu_2$	42.95	1.80	28.02	0.0	42.96	2.57	26.95	2.1

[a] Carbon and hydrogen analyses were done by the Weiler and Strauss Microanalytical Laboratory, Oxford, England.
[b] The water content was determined by loss of weight on vacuum (2–5 mm.) drying at 100° over phosphorus pentoxide.

TABLE IV.—*In Vivo* ANTITUBERCULAR ACTIVITIES OF THE RIBOFLAVIN-5′-PHOSPHATE CHELATES[a]

Compound	In Diet, %	No. of Mice	Mortality, %	Mean Survival Time, Days[b]
R_2-Cu_2·$4H_2O$	0.1	5	60	20.5+
R_2-Zn·$5H_2O$	0.02	5	60	19.8+
R_2-Co·$3H_2O$	0.1	5	60	20+
R_2-Fe_3·$6H_2O$	0.2	5	80	19+
INH[c]	0.01	5	0	21+
Controls	..	10	70	19.7+

[a] Determined at the Lilly Research Laboratories by W. B. Sutton using *M. tuberculosis* $H_{37}Rv$ in mice.
[b] A + indicates that the surviving animals were sacrificed at the indicated times for examination of lesions.
[c] INH = isonicotinyl hydrazide.

acid solution containing 0.03–0.04 Gm. of cobalt per sample was prepared by treating the sample first with 5 ml. of concentrated hydrochloric acid and heating to break the complex, and then adding 195 ml. of water. The solution was heated to about 80°, and approximately 0.5 Gm. of α-nitroso-β-naphthol in 20 ml. of hot, freshly prepared 50% acetic acid was added for every 0.01 Gm. of cobalt present. The precipitate settled, and more reagent was added until it was certain that precipitation was complete. The precipitate was allowed to stand for two hours and was then transferred to a crucible, washed with hot water, and heated slowly to remove carbon. Ignition was carried out in an oxidizing atmosphere at 800–900°, and the residue was weighed as Co_3O_4.

Nickel.—This procedure was modified from that of Hillebrand, *et al.* (14). A solution containing 0.02–0.03 Gm. of nickel was prepared by first treating the complex with 15 ml. of 10% hydrochloric acid and then adding 0.5 Gm. of tartaric acid and 185 ml. of water. It was heated nearly to boiling, sufficient concentrated ammonium hydroxide was added to bring the pH to 7, and it was then made slightly acid with 5% hydrochloric acid. At least 5 ml. of a 1% alcoholic solution of dimethylglyoxime was then added for every 0.01 Gm. of nickel present. Immediately, sufficient dilute (10%) ammonium hydroxide was added dropwise to give a slight excess beyond the point of precipitation. The precipitate was digested on a steam bath for one hour, filtered in a sintered-glass crucible, and washed with hot water. It was dried at 150° for forty-five minutes and weighed as $C_8H_{14}N_4O_4Ni$.

Copper.—A modification of the method of Hillebrand, *et al.* (15), was used. A sample containing from 0.05–0.25 Gm. of copper was treated with 20 ml. of acetic acid to decompose the chelate and then

with 80 ml. of water. To the solution was added 5 Gm. of potassium iodide dissolved in a little water, and 75 ml. of carbon tetrachloride was added. The mixture was filtered into a separatory funnel, the filtration funnel being washed with carbon tetrachloride, and the filtrate was immediately titrated with thiosulfate solution, preferably having a titer equal to 0.005 Gm. of copper. The funnel was shaken after each addition of thiosulfate; the end point was the disappearance of the pink color in the organic layer. The usual method of titration of the aqueous solution could be employed if the chelate did not impart color to the solution.

Zinc.—A modification of the method of Kolthoff and Sandell (16) was used. A solution containing approximately 0.05–0.1 Gm. of zinc was prepared by treating the compound with 3 ml. of concentrated hydrochloric acid and then diluting with 200 ml. of water. To the solution was added a few drops of methyl red followed by ammonium hydroxide until the solution just turned yellow. It was heated to 90°, and 30 ml. of 20% diammonium phosphate was slowly added. It was kept hot on a steam bath for an additional half-hour, and the precipitate was set aside for two hours. The product was filtered through a porcelain crucible, washed with 1% diammonium phosphate until chlorine-free, and then washed with 50% alcohol. It was heated slowly until water and ammonia were expelled, and was then heated at 900° to constant weight. It was weighed as $Zn_2P_2O_7$.

Iron.—The procedure of Kolthoff and Sandell (17) was used without modification.

SUMMARY

1. Metal complexes, which are probably chelates, have been prepared of riboflavin-5′-phosphate and phthaloylhydrazine using divalent copper, cobalt, nickel, iron, and zinc ions.

2. Analytical results on the riboflavin-5′-phosphate chelates show the following general formulas to exist at three pH values: at pH9, $R\text{-}M_4\cdot xH_2O$ for Co and Ni chelates; at pH7, $R_2\text{-}M_3\cdot xH_2O$ for Co, Ni, Fe, and Zn chelates, and $R_2\text{-}M_5\cdot xH_2O$ for Cu; at pH3, $R_2\text{-}M\cdot xH_2O$ for Co, Ni, and Zn chelates, and $R_2\text{-}M_2\cdot xH_2O$ for Cu.

3. Antitubercular tests in mice carried out on a number of these chelates showed them to be essentially inactive, although small increases in survival time were noted with the riboflavin-5′-phosphate chelates.

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The Preparation of Some Derivatives of Oximes*

By LOUIS GASS† and FRANK W. BOPE

Fourteen new esters of a series of oximes have been synthesized. Some of the more important physical properties have been determined.

OXIMES and their derivatives have been found to possess activity as muscle relaxants (1, 2), local anesthetics (3) cholinesterase activators (4), antifungal agents (5, 6, 7), trypanocides (8), antibacterials (9, 10), tuberculostatics (11, 12), and antirickettsials (13). Several oximes have been shown to possess anticonvulsant activity, comparable to diphenylhydantoin, against electric shock (14). However, they also produced certain toxic effects (1, 15–17).

Little work has been done in the field of esters of oximes for the preparation of useful products with the exception of those having insecticidal activity (18). It was, therefore, decided to prepare a series of esters of oximes for later pharmacological testing, having possible anticonvulsant activity similar to the original oximes.

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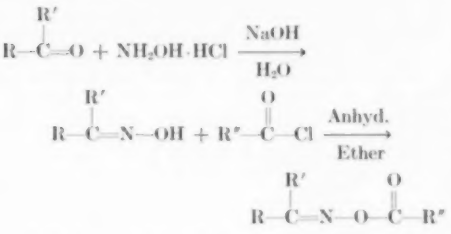
This material was abstracted from a portion of a dissertation submitted to the Graduate School of The Ohio State University by L. Gass in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The synthesis of a series of selected esters of five oximes, namely: anisaldoxime, benzaldoxime, acetophenone oxime, benzophenone oxime, and cyclohexanone oxime, is reported herein.

The method of synthesis of these esters was essentially the same as that of a standard esterification except an oxime was used in place of the alcohol. A series of acids in the form of acid chlorides was used to make the esters of each oxime as follows:



Only the syn (α) oximino isomers of the oximes having isomers were prepared since they are more stable than the anti-(β) isomers (19, 20).

EXPERIMENTAL

Synthesis of the Oximes

Cyclohexanone oxime was prepared according to the method of Vogel (21). Recrystallization from petroleum ether resulted in a yield of 80.7% of the oxime melting at 92–93°.

Benzophenone oxime was prepared according to the method described in "Organic Syntheses" (22). The crude product was recrystallized from methanol to give a 78.5% yield of the oxime melting at 144–145°.

syn-Benzaldoxime was prepared by a procedure given by Vogel (23) in a yield of 75.6% with a melting point of 33–34°.

syn-*p*-Methoxy benzaldoxime was prepared by the method used for *syn*-benzaldoxime. The resulting impure product was recrystallized from ethanol in a 75.0% yield melting at 63–64°.

syn-Acetophenone oxime was also prepared by the method used for *syn*-benzaldoxime. The crude product was recrystallized from water in a yield of 80.5% and melting at 58–60°.

Synthesis of Esters of the Oximes

The following general procedure was used in preparing all esters of the oximes with the exception of the two listed below where the procedure was modified in order to facilitate purification or crystallization. Analytical data for each compound are listed in Table I.

In a 125-ml. Erlenmeyer flask fitted with a magnetic stirrer and cooled in an ice bath was placed 0.05 mole of the oxime and 50 ml. of anhydrous ether to which was added 0.05 mole of the appropriate acid chloride.[1] The mixture was stirred for five minutes after the addition of the acid chloride and the flask was then removed from the ice bath. Upon evaporating the ether under a stream of air, a precipitate formed which was mixed with 200 ml. of a 3% solution of sodium bicarbonate and stirred for ten minutes. The solid material was then filtered with suction, washed with 300 ml. of cold distilled water, and then recrystallized from an ethanol-water mixture after decolorizing with activated charcoal. The recrystallized products were dried *in vacuo* (2 mm. over phosphorus pentoxide) from 40 to 60°.

Cyclohexanone Oxime Phenyl Acetate.—Using the general procedure described above, 5.7 Gm. (0.05 mole) of cyclohexanone oxime was mixed with 7.7 Gm. (0.05 mole) of phenyl acetyl chloride in anhydrous ether. The ether was evaporated under a stream of air and the liquid remaining was dissolved in 100 ml. of benzene. After washing the benzene solution twice with 100-ml. portions of 3% sodium bicarbonate solution, the benzene layer was dried over anhydrous sodium sulfate. This dried benzene solution was decolorized with activated charcoal and the benzene removed under a stream of air. The remaining oil-like product was dried over phosphorus pentoxide in a vacuum desiccator. Several attempts were made to purify the oil-like product by crystallization but without success. When purification was attempted by distillation, the oil-like product distilled at 80°/2 mm. with decomposition.

***syn*-Acetophenone Oxime Phenyl Acetate.**—The same procedure above was used to react 6.8 Gm. (0.05 mole) of *syn*-acetophenone with 7.7 Gm. (0.05 mole) of phenyl acetyl chloride and purify the resulting ester. The oil-like product obtained from benzene was dissolved in 100 ml. of absolute ethanol and decolorized with activated charcoal. The filtrate was placed in a dry ice-acetone bath and distilled water was added slowly with rapid stirring until a precipitate formed. The crystals were filtered with suction and dried in a vacuum desiccator over phosphorus pentoxide in a refrigerator.

Infrared Spectra.—Infrared spectra of the oximino compounds were prepared in the Department of Chemistry of The Ohio State University using a Baird Associates Infrared Recording Spectrophotometer, Model B, equipped with a sodium chloride prism. A potassium bromide pellet of each compound served as the investigated sample since there were no bands present which interfered with the absorption peaks of the oximino compounds. Due to the complexity of the compounds studied, only two characteristic bands were considered; the —C=N— band appearing at 6.02 to 6.22 μ (24) and the —C(=O)—O— band appearing at 5.7 to 5.85 μ.[2] These two characteristic bands were present in the infrared spectra of all the esters prepared.

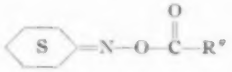
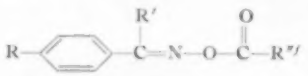
DISCUSSION

Anhydrous ether was used in preference to other solvents for the esterification of the oximes similar to

[1] All acid chlorides were used *per se* with the exception of *p*-nitro benzoyl chloride which was dissolved in anhydrous ether (25 ml.) before adding to the appropriate oxime.

[2] Ester carbonyl absorption band was taken from a compilation of known groups by the Ohio State University Department of Chemistry.

TABLE I.—ESTERS OF OXIMES

R	R′	R″[c]	Drying[a] Temp. °C.	Yield, %	M. P.[b] °C.	Molecular Formula	Nitrogen, % Calcd.	Nitrogen, % Found[d]	Saponification Equivalents Calcd.	Saponification Equivalents Found No. 1	Saponification Equivalents Found No. 2
											
		—C_6H_4—NO_2	60	95.4	111–112	$C_{15}H_{14}O_4N_2$	10.69	10.86	262.2	266.1–265.1	
		—C_6H_4—OCH_3	40	81.1	71–72	$C_{16}H_{17}O_3N$	5.67	5.63	247.2	250.1–247.7	
		—CH_2—C_6H_5		60.6	80°/2 mm.[e]	$C_{15}H_{17}O_2N$	6.06	6.01	231.2	231.2–227.3	
											
H—	—C_6H_5	—C_6H_4—NO_2	60	83.9	153–155	$C_{20}H_{14}O_4N_2$	8.09	7.98	346.2	343.9–347.8	
H—	—C_6H_5	—C_6H_4—OCH_3	60	81.0	157–159	$C_{21}H_{17}O_3N$	4.23	4.33	331.2	331.1–338.2	
H—	—C_6H_5	—CH_2—C_6H_5	40	85.1	79–81	$C_{21}H_{17}O_2N$	4.44	4.51	315.2	316.9–317.5	
H—	—H	—C_6H_4—NO_2	60	81.5	166–168	$C_{14}H_{10}O_4N_2$	10.37	10.37	270.1	267.7–275.7	
H—	—H	—CH_2—C_6H_5	40	75.3	70–71	$C_{15}H_{13}O_2N$	5.86	6.02	239.2	233.8–234.4	
CH_3O—	—H	—C_6H_4—NO_2	60	63.3	161–163	$C_{16}H_{12}O_5N_2$	9.33	9.34	300.2	300.0–297.4	
CH_3O—	—H	—CH_2—C_6H_5	40	74.3	55–57	$C_{16}H_{15}O_3N$	5.20	5.32	269.2	264.8–272.2	
H—	—CH_3	—C_6H_5	60	83.7	100–102	$C_{15}H_{13}O_2N$	5.86	5.92	239.2	240.8–241.4	
H—	—CH_3	—C_6H_4—NO_2	60	77.5	167–169	$C_{15}H_{12}O_4N_2$	9.86	9.86	284.2	280.1–279.5	
H—	—CH_3	—C_6H_4—OCH_3	60	74.3	90–92	$C_{16}H_{15}O_3N$	5.20	5.13	269.2	269.2–270.7	
H—	—CH_3	—CH_2—C_6H_5	5	31.6	166–169	$C_{16}H_{15}O_2N$	5.53	5.35	253.2	253.2–251.8	

[a] *In vacuo*, 2 mm., over P_2O_5. [b] Melting points taken on Fisher-Johns Apparatus uncorrected. [c] All substitutions on the aromatic rings are in the *p*-position. [d] Nitrogen analyses performed by Galbraith Laboratories, Knoxville, Tenn. [e] Boiling point with decomposition. [f] All unsymmetrical compounds were the *syn* (α) forms.

the method used by Minunni (25, 26) when it was found that the yield of the final products were higher from this solvent than any other used and that it favored the formation of the *syn*-esters (27, 28). Attempts were made to carry out the esterification of the oximes in dimethylformamide, pyridine, and pyridine-ether mixture. With cyclohexanone oxime and benzophenone oxime, the yields of the esters formed ranged from 25 to 40% lower in these solvents than in pure ether. Hauser and Vermillion (29, 30) similarly prepared benzoate esters of oximes and found that the *syn* forms were stable in pyridine alone or in a pyridine-triethyl or propylamine mixture. However, in the presence of pyridine and pyridinium chloride, formed during esterification, there was a decomposition of the *syn*-oximes into the anti form and then into the nitrile if the product remained more than a few minutes in this solvent. In several cases it was found that esterification of oximes led to the formation of the *syn*- and anti-esters (27, 29), while in another instance esterification was found to take place by the combination of a lactone with an oxime in the presence of triethyl amine (31).

Grammaticakis (32, 33) suggested that N-acylation is favored over O-acylation by showing that acetylation of benzaldoxime yielded the same product as the oxidation of N-acetyl-N-benzylhydroxylamine, namely, N-acetylbenzaldoxime. Also absorption spectra studies of acetylated oximes showed the presence of the nitronic structure which favored the N-acetylation. This was also confirmed by the reaction of substituted arylaldoximes with the Grignard reagent since the Grignard reacts only with the

$$-\overset{|}{C}=N-$$

group of these compounds. The above data substantiated a previous claim of Schmidt (34) that N-acylation took place in preference to O-acylation.

Exner (35) challenged the above work on the basis of his experimental results with the same types of compounds. Ultraviolet spectral analyses which he carried out corresponded to the O-acyl derivatives and the reduction of these O-acyl derivatives with lithium aluminum hydride gave primarily the corresponding alcohol and oxime which was further reduced to the amine.

Our infrared analyses substantiate Exner's work.

$$-\overset{|}{C}=N-$$

They show the band, as stated previously, appearing in the correct range, 6.02 to 6.22 μ and the

$$-\overset{\overset{O}{\|}}{C}-O-$$

band, 5.7 to 5.85 μ, indicative of an ester linkage. This evidence together with the nitrogen analyses favors the existence of O-acylation rather than N-acylation.

SUMMARY

Fourteen new esters of a series of oximes, namely: the benzoates, *p*-nitro-benzoates, *p*-methoxy-benzoates and phenyl acetates of cyclohexanone oxime, benzophenone oxime, benzaldoxime, anisaldoxime, and acetophenone oxime are reported.

The esters are characterized by nitrogen analyses, saponification equivalents, and infrared analyses which favor the existence of O-acylation rather than N-acylation.

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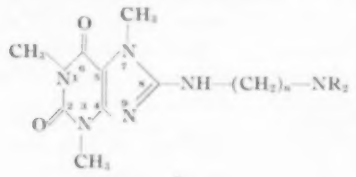
8-(Dialkylaminoalkylamino)-caffeines as Potential Pharmacodynamic Agents*

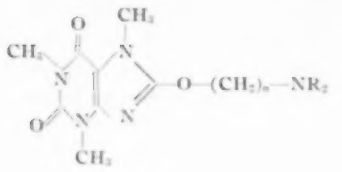
By J. H. BURCKHALTER and DALE R. DILL

A group of caffeine and theophylline derivatives containing basic side chains at position 8 were synthesized as potential pharmacodynamic agents. An attempt was made to explain why type I compounds were at times isolated as the free base and again as the hydrochloride and also why the dihydrochloride and dimethiodide of the same compounds could not be prepared. As an illustration of pharmacodynamic actions, the methiodide of one of the type I compounds caused marked postural hypotension in the absence of autonomic blockade, while no ganglionic blocking or anticholinergic effect was observed.

IN A SEARCH for chemical structures which might possess hypotensive action, it was considered desirable to attach basic side chains to nuclei which are well known for their pharmacodynamic properties. As a part of these studies, caffeine was chosen since it is known to possess a variety of cardiovascular activities, and, since the reactive 8-chlorocaffeine is readily available (1), it was decided to synthesize a number of 8-dialkylaminoalkylaminocaffeines (I) and 8-dialkylaminoalkoxycaffeines (II). Also



I



II

their quaternary ammonium derivatives were desirable because of the well established activity of quaternary nitrogen compounds against autonomic functions in the body.

The preparative method employed for I was similar to that of Blicke and Godt (2), who made certain 8-alkylaminocaffeines by heating an alcoholic mixture of 8-chlorocaffeine and alkylamines in a pressure bottle. For the purpose of these studies, diamines were used in place of monoamines and most of the results are summarized in Table I together with a number of quaternary ammonium derivatives. Analogous compounds have been made by others by sealed-tube reactions (3).

When ethylenediamine was employed in the reaction designated as Procedure I, 8-(ethylenediamino)-bis-caffeine (V), which contains two caffeine residues, was obtained. When 8-chlorocaffeine was replaced by 8-chlorotheophylline in Procedure I and employed with the appropriate diamine, 8-[4-(1-methylpiperazinyl)]-theophylline (VI) and 8-(N,N-diethylethylenediamino)-theophylline hydrochloride (VII) were synthesized. With N,N-dimethylethylenediamine, 8-chlorotheophylline yielded directly the 8-chlorotheophyllate salt (VIII) instead of the hydrochloride or free base.

During the preparation of this manuscript, a publication appeared which describes the synthesis of three 8-(dialkylaminoalkoxy)-caffeines of structure II (4). For this reason, we wish to report now the synthesis of three compounds of the same general structure.

Although Cooper and Cheney (5) and Chakravarty and Jones (4) were successful in condensing 8-chlorocaffeine with the sodium salts of amino alcohols to obtain type II compounds, we were unsuccessful in employing this approach. Instead, 8-chlorocaffeine, ethylene bromohydrin, and sodium acetate were heated at elevated temperature to give 8-(2-bromoethoxy)-caffeine which was allowed to react with dimethylamine, pyrrolidine, and N-methylpiperazine to produce the expected products (II) in excellent yield.

When N,N-dimethylethylenediamine was employed according to Procedure I, a caffeine derivative of type I (compound 1 of Table I) was obtained in seven different experiments as the free base. This result was expected, especially since a one-hundred per cent excess of starting diamine was employed in order to take up the hydrogen

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TABLE I. 8-(DIALKYLAMINOALKYLAMINO)-CAFFEINES

No.	R	Procedure	M. P., °C.	Yield, %	Formula	Analyses C Calcd.	C Found	H Calcd.	H Found
1	N,N-Dimethylethylenediamino	I	205	89	$C_{12}H_{20}N_6O_2$[a,b]	51.41	51.24	7.19	7.09
2	N,N-Dimethylethylenediamino hydrochloride	I	289	44	$C_{12}H_{20}N_6O_2 \cdot HCl$[a,c,d,e,i]	45.89	45.52	6.69	6.75
3	N,N-Dimethylethylenediamino methiodide	III	285	80	$C_{14}H_{23}N_6O_2I$[a,e,p,r]	36.97	36.78	5.44	6.57
4	N,N-Dimethylethylenediamino *p*-chlorobenzyl chloride	III	236	90	$C_{19}H_{30}N_6O_2Cl_2 \cdot 2H_2O$[f,r,t,u]	47.80	48.12	6.33	6.29
5	N,N-Diethylethylenediamino	I	186	71	$C_{14}H_{24}N_6O_2$[a,b]	54.52	54.28	7.84	7.56
6	N,N-Diethylethylenediamino hydrochloride	I,IIA	270	76,98	$C_{14}H_{24}N_6O_2 \cdot HCl$[a,c,f]	48.75	48.88	7.31	7.55
7	N,N-Diethylethylenediamino ethiodide	III	264	73	$C_{16}H_{25}N_6O_2I$[q,r,s]	41.38	41.30	6.39	6.34
8	N-Methylpiperazinyl hydrochloride	I	344	65	$C_{14}H_{20}N_6O_2 \cdot HCl$[d,g,h]	47.48	47.83	6.44	6.43
9	N,N-Dimethyl-1,3-propanediamino	I	178	61	$C_{11}H_{22}N_6O_2 \cdot H_2O$[g,i,k]	51.46	51.46	7.64	7.68
10	N,N-Dimethyl-1,3-propanediamino methiodide	III	279	90	$C_{14}H_{26}N_6O_2I$[l,m,r]	38.54	38.59	5.78	5.83
11	N,N-Diethyl-1,6-hexanediamino[w]		183	48	$C_{18}H_{32}N_6O_2 \cdot {}^1/_2H_2O$	57.88	58.26	8.97	8.88
12	N,N-Diethyl-1,6-hexanediamino hydrochloride	IIB	236	94	$C_{18}H_{32}N_6O_2 \cdot HCl$[v]	53.92	53.95	8.30	8.32
13	N,N-Diethyl-1,6-hexanediamino ethiodide	III	185	99	$C_{20}H_{37}N_6O_2I \cdot H_2O$[p,s]	44.75	44.61	7.06	7.30

[a] Recrystallized from absolute alcohol three times. [b] In one experiment the hydrochloride of this base was also obtained from the reaction. [c] Heated at 190–200°. [d] Yields may be improved by obtaining second crop crystals. [e] Hydrochloride salt of the free base is less soluble in absolute alcohol than is the free base. [f] Principal product from reaction. [g] Heated at 150°. [h] From 95% alcohol. [i] Second crop product may be free base. [j] From Skelly Solve B-absolute alcohol mixture. [k] Crude product was treated with dilute sodium hydroxide and extracted with chloroform which was then evaporated. [l] Heated at reflux for an hour. [m] From methyl alcohol. [n] Decomposed by light. [o] From methyl alcohol or water-methyl alcohol. [p] Heated at reflux overnight. [q] From alcohol-water mixture. [r] Washed with ether. [s] Refluxed for sixty hours. [t] Hygroscopic. [u] From alcohol. [v] From isopropyl alcohol. [w] See experimental.

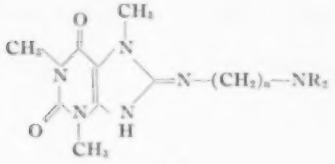
chloride produced. However, during one experiment, when the temperature of reaction was elevated to 190–200°, compound 1 was isolated, surprisingly, as the monohydrochloride salt. Further, when N,N-dimethyl-1,3-propanediamine or N-methylpiperazine was used in the same reaction at 150°, the product precipitated in the form of the hydrochloride salt. The last result appears strange in view of the fact that a quantity of the hydrochloride of 8-(N-methylpiperazinyl)-caffeine was insoluble in alcohol at room temperature, but dissolved upon treatment with N-methylpiperazine. Finally, in one experiment involving N,N-diethylethylenediamine, the product was isolated as the free base, while in another experiment under apparently the same conditions the hydrochloride was isolated.

In an attempt to find an explanation for these results, relative pH determinations using the type I bases and the starting diamines in alcoholic solution were made. However, no light was shed thereby upon the results obtained, for, as expected, lower readings were noted for the product than for the starting amine. An explanation based upon chance precipitation was next considered.

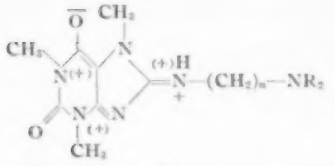
Since a hundred per cent excess of starting amine was employed in the syntheses, at the end of the reaction four compounds should be in equilibrium. They are starting amine, product amine, and their hydrochlorides. Once one of the constituents, for example the product amine, has through chance commenced to separate from solution, the equilibria involving the four compounds would shift to favor removal of hydrogen chloride from the product by the excess of starting amine until a good yield of free base of the product was isolated. Likewise, if the product happened to begin to crystallize as the hydrochloride the equilibria would shift so that the starting amine would give up its hydrogen chloride.

Product amine + starting amine HCl ⇌
Product HCl + starting amine

When an excessive amount of hydrogen chloride was passed into a solution of a type I base (Procedure II), a dihydrochloride salt might have been expected, but only the monohydrochloride resulted. Likewise, attempts to make bis quaternaries (Procedure III) gave the monosubstituted derivatives. Further, neither 8-N-pyrrolidinyl- nor 8-N-piperidinylcaffeine, originally made by Blicke and Godt (2), could be quaternized. These facts suggest that the amino group at position 8 of caffeine derivatives of I is lacking in basicity.



III



IV

Structure III, a tautomeric form of I, does not allow as many resonance structures as I nor does it provide the conjugated system of I. Thus, there would be a large loss in resonance energy in going from structure I to III. For these reasons, only structure I is considered to be of importance, and taking a closer look at it and its resonance forms, one can see the 6-oxo atom as the major perturbation. Because of this carbonyl, one can designate the resonance forms of I as IV. Not only is high resonance energy demonstrated by IV, but also it possesses an excellent conjugated system. If the secondary 8-amino group were to be involved in the formation of a hydrochloride salt, some resonance structures would be prevented and resonance energy would be lowered. Further, it may be observed that I is a vinylogous amide, and, therefore, the ring nitrogen at position 9 as well as the secondary amino at 8 would be lacking in basic properties. Thus, an explanation is offered for the inability of I to form dihydrochloride and dimethiodide salts under the conditions employed.

Pharmacological Results.—After having studied intravenously the action of several of the caffeine derivatives of Table I in anesthetized, vagotomized dogs, Dr. Neil C. Moran, of the National Heart Institute, Bethesda, Md.,[1] made the following statement: ". . .the compound of most interest is compound 3 because of the marked postural hypotension in the absence of autonomic blockade. Also, compounds 1 and 4 look interesting because of the direct fall in blood pressure in the absence of autonomic blockade. Whether these actions can be attributed to a central nervous system effect can only be determined by further, more definitive experiments."

Dr. Graham Chen, of Parke, Davis and Co. Research Laboratories, Detroit, Mich., found compounds 3 and 8 of Table I, 8-[2-(1-pyrrolidinyl)-ethoxy]-caffeine (IX) and 8-[2-(4-methyl-1-piperazinyl)-ethoxy]-caffeine (X) to be devoid of hypotensive activity when administered orally at 20 mg./Kg. in the perinephritic rat. Also, compounds 3 and X were found to lack hypnotic activity when administered orally at 500 mg./Kg. in the rat. Concerning compound 3, Dr. Chen added, "No ganglionic blocking or anticholinergic effect was observed in pentobarbitalized dogs at a total dose of 10 mg./Kg. intravenously. The intraperitoneal lethal toxicity is about 60 mg./Kg. in mice."

EXPERIMENTAL

8-Chlorocaffeine.—This was prepared by Long's modification of Fischer and Reese's procedure (1). In this method, dry caffeine was dissolved in chloroform and chlorine gas was passed through the solution until the precipitate which first appeared had dissolved. After removal of the chloroform, the desired product in very pure state remained, m. p. 184°. In the present studies, it was found that it was very important to employ anhydrous caffeine. Nearly a quantitative yield of 8-chlorocaffeine was obtained when the commercially available caffeine monohydrate was dried at 60° for three days before use.

8-Chlorotheophylline.—This was prepared by the method of Y. Yoshitomia (6).

N,N-Diethyl-1,6-hexanediamine.—This is the only required diamine which was not readily available from commercial sources. It was prepared from ε-bromocapronitrile and diethylamine by the procedure of Breslow and Hauser (7). The resulting amino nitrile was reduced with Raney nickel to the desired product using the method of Turner (8).

Procedure I.—By a procedure similar to that of Blicke and Godt (2), 0.05 mole of 8-chlorocaffeine or 8-chlorotheophylline was mixed with 0.10 mole of the appropriate diamine and 75 ml. of absolute alcohol in a citrate pressure bottle and heated at 170° for twenty-four hours. After cooling to room

[1] Present address: Emory University, Ga.

temperature, the pressure bottle was opened and the contents were refrigerated overnight. The product was obtained by filtration and frequently purified by recrystallizing from absolute alcohol and washing with anhydrous ether.

Procedure II.—(*A*) The free base of the diamine (0.01 mole) was dissolved in 100 ml. of hot absolute alcohol and, while the solution was still hot, a vigorous stream of dry hydrogen chloride gas was passed into it until it was quite acidic. The volume was then reduced by one-half and the mixture was refrigerated. The product was filtered from the solution and then recrystallized from absolute alcohol.

(*B*) The free base of the diamine (0.01 mole) was dissolved in anhydrous ether and a vigorous stream of dry hydrogen chloride gas was passed into the solution. The hydrochloride salt immediately precipitated. The product was collected on a filter and recrystallized from isopropyl alcohol.

Procedure III.—The appropriate diamine (0.01 mole) was dissolved in 75 ml. of boiling anhydrous benzene. Then, the appropriate alkyl halide (0.04 mole) was added and heating was continued as noted in Table I. The mixture was chilled, and the product obtained by filtration was recrystallized and washed with anhydrous ether. The compounds were then dried in the oven at 60° for twenty-four hours. The quaternary salts were frequently unstable to light and hence must be stored in colored bottles to prevent decomposition.

8 - (N,N - Diethyl - 1,6 - hexanediamino) - caffeine (Compound 11, Table I).—To a citrate bottle was added 8.6 Gm. (0.05 mole) of N,N-diethylhexane-1,6-diamine, 12.3 Gm. (0.05 mole) of 8-chlorocaffeine, and 80 ml. of absolute alcohol. After heating at 160° for thirty-four hours, the mixture was chilled. The solid which precipitated was removed by filtration; it proved to be 4.8 Gm. of unreacted 8-chlorocaffeine. The filtrate was evaporated to dryness and the residue was recrystallized twice from isopropyl alcohol. This solid was then mixed and heated on the steam bath with a concentrated sodium hydroxide solution, and extracted with benzene which was then evaporated. The product was purified by three recrystallizations from toluene, washed with Skelly B, and then dried at 50° for forty-eight hours. The yield was 9.0 Gm. (48%), m. p. 182–183°.

8-(Ethylenediamino)-bis-caffeine (V).—This compound was prepared by Procedure I except that equimolar portions of 8-chlorocaffeine (24.6 Gm., 0.1 mole) and ethylenediamine (5.20 Gm., 0.1 mole) were heated together in a citrate bottle at 150° for twelve hours. After cooling, filtering, washing with alcohol, and recrystallizing twice from dimethylformamide, the product was obtained. The yield was 17.3 Gm. (64%), m. p. > 360°.

Anal.—Calcd. for $C_{18}H_{26}N_{10}O_4$: C, 48.64; H, 5.44. Found: C, 49.23; H, 5.39.

8 - [4 - (1 - Methylpiperazinyl)] - theophylline (VI).—Procedure I was used for the preparation of this compound with the substitution of 8-chlorotheophylline for 8-chlorocaffeine. The product was recrystallized three times from dioxane. After drying for twenty-four hours at 60°, a yield of 9.0 Gm. (65%) was obtained, m. p. 275–276°.

Anal.—Calcd. for $C_{12}H_{18}N_6O_2$: C, 51.78; H, 6.52. Found: C, 52.20; H, 6.31.

8 - (N,N - Diethylethylenediamino) - theophylline Hydrochloride (VII).—8-Chlorotheophylline was substituted for 8-chlorocaffeine in Procedure I. The crude product was recrystallized from absolute ethyl alcohol yielding 14.5 Gm. (88%), m. p. 162°.

Anal.—Calcd. for $C_{12}H_{23}ClN_6O_2$: C, 47.19; H, 7.00. Found: C, 47.46; H, 6.92.

8 - (N,N - Dimethylethylenediamino)-theophylline 8-Chlorotheophyllate (VIII).—A mixture of 10.7 Gm. (0.05 mole) of 8-chlorotheophylline, 8.8 Gm. (0.1 mole) of N,N-dimethylethylenediamine and 50 ml. of absolute ethyl alcohol was heated in a citrate bottle at 150° for fifteen hours. After cooling and filtering, the product was recrystallized three times from absolute ethyl alcohol to yield 12.0 Gm. (92%), m. p. 243–244°.

Anal.—Calcd. for $C_{18}H_{28}ClN_{10}O_4$: C, 41.80; H, 5.04. Found: C, 41.80; H, 5.07.

When an alcoholic solution of the product was treated with hydrogen chloride gas, 8-chlorotheophylline was liberated, as indicated by mixed melting point and C-H analysis.

8-(2-Bromoethoxy)-caffeine.—A mixture of 12.3 Gm. (0.05 mole) of 8-chlorocaffeine, 8.2 Gm. (0.1 mole) of sodium acetate and 25 ml. (0.35 mole) of ethylene bromohydrin was placed in a citrate bottle and heated at 190° for forty-eight hours. The contents of the bottle were chilled overnight in the refrigerator. The product was removed by filtration and then purified by three recrystallizations from absolute alcohol. (The filtrate from the reaction may be used with more 8-chlorocaffeine in repeating the experiment.) After decolorizing with charcoal a yield of 14.0 Gm. (83%) was obtained, m. p. 198–199°.

Anal.—Calcd. for $C_{10}H_{13}BrN_4O_3$: C, 37.87; H, 4.13. Found: C, 37.37; H, 3.94.

8-[2-(1-Pyrrolidinyl)-ethoxy]-caffeine (IX).—A mixture of 3.0 Gm. (0.0095 mole) of 8-(β-bromoethoxy)-caffeine, 15 ml. (0.18 mole) of pyrrolidine, and 40 ml. of anhydrous benzene was heated at reflux temperature for twenty hours. After cooling, the product precipitated and was removed by filtration. This solid was extracted with ether. The ether was removed and the residue was recrystallized three times from absolute alcohol and dried at 60° for twenty-four hours yielding 2.8 Gm. (93%) of product, m. p. 189–190°.

Anal.—Calcd. for $C_{14}H_{21}N_5O_2$: C, 54.71; H, 6.89. Found: C, 54.68; H, 6.75.

8 - [2 - (4 - Methyl - 1 - piperazinyl) - ethoxy]-caffeine (X).—To a mixture of 3.0 Gm. (0.0095 mole) of 8-(2-bromoethoxy)-caffeine in 40 ml. of anhydrous benzene, 15 ml. of N-methylpiperazine was added and the solution was heated at reflux temperature for twenty-four hours. After refrigerating for three days, an insoluble material was filtered from the solution. A total volume of 50 ml. of benzene and excess N-methylpiperazine was removed by distillation under reduced pressure. While hot, the residue was mixed with 25 ml. of absolute alcohol. After cooling, crystallization was induced by scratching the side of the beaker with a glass rod. The product was removed by filtration and purified by three recrystallizations from absolute alcohol. It was dried at 60° for twenty-four hours. Second crop crystals were obtained by reducing the volume of the filtrate and cooling to give 3.0 Gm. (91%) of product, m. p. 144–145°.

Anal.—Calcd. for $C_{15}H_{24}N_6O_3$: C, 53.55; H, 7.19. Found: C, 53.51; H, 6.93.

8-(2-Dimethylaminoethoxy)-caffeine (XI).—In a citrate pressure bottle which had been chilled in a sodium chloride ice bath, there was added 2.0 Gm. (0.0063 mole) of 8-(2-bromoethoxy)-caffeine and 30 ml. of anhydrous dimethylamine which had also been chilled. (Bottle should be wrapped as a precaution.) The pressure bottle was sealed and the mixture was allowed to stand at room temperature for twenty-four hours. After having been chilled to a temperature below 0°, the bottle was unsealed and allowed to stand open to the air for twenty-four hours. The solid remaining was recrystallized three times from absolute alcohol and was decolorized once with charcoal. The product thus obtained after drying at 60° for twenty-four hours weighed 1.5 Gm. (85%), m. p. 211°.

Anal.—Calcd. for $C_{12}H_{19}N_5O_3$: C, 51.23; H, 6.81. Found: C, 51.05; H, 6.55.

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Micromethod for the Estimation of Anesthetics Derived From Aminobenzoic Acid*

Procaine Hydrochloride[1] and Tetracaine Hydrochloride[2] Combined in Cartridges for Dental Anesthesia

By MORRIS E. AUERBACH and MURRAY M. TUCKERMAN†

From a 1-ml. sample, total anesthetic is computed from the ultraviolet absorption of the extracted anesthetic bases in strongly acid solution. The minor constituent, tetracaine hydrochloride, is determined by simultaneously diazotizing the procaine and nitrosating the tetracaine. A salt, soluble in water, is produced from the procaine diazonium chloride by coupling it quantitatively with Chicago acid. The nitroso-tetracaine is then extracted from the alkaline medium with chloroform and determined spectrophotometrically.

In 1957 Pratt (1) reported a spectrophotometric titration with nitrous acid for the estimation of procaine hydrochloride, propoxycaine hydrochloride, tetracaine hydrochloride, and the combination of procaine hydrochloride with tetracaine hydrochloride. For the commercially available formula[3] containing 20.0 mg. procaine hydrochloride and 1.5 mg. tetracaine hydrochloride per ml., Pratt required a 20-ml. sample. The method presented below requires a 1-ml. sample of the same formulation. Since this formulation is packed in 2-ml. doses, the method allows the estimation of the anesthetics in the individual cartridges.

PROCEDURE

Reagents and Apparatus.—Sodium carbonate solution, 10%; chloroform; alcoholic hydrogen chloride, 7 *M* (prepared by absorbing hydrogen chloride gas in alcohol); sulfuric acid, 7 *N*; sodium nitrite, 7%; hydrochloric acid, 1 *N*; Chicago acid (1-amino-8-naphthol-2,4-disodium sulfonate) recrystallized by the method of English (2), 2%.

Apparatus includes: Beckman DU spectrophotometer or equivalent.

Extraction of Anesthetic Bases.—Transfer a 1.00-ml. sample to a separatory funnel containing 20 ml. of water. Add 2 ml. of 10% sodium carbonate solution, mix. Extract with three 25-ml. portions of chloroform, pooling the chloroform extracts in a separatory funnel. Wash the pooled chloroform extracts with 15 ml. of water. Filter the washed chloroform extracts through creped filter paper into an evaporating dish, add 0.2 ml. of 7 *M* alcoholic hydrogen chloride, evaporate to dryness. Take up residue in water to make 100.0 ml.

This extraction separates the anesthetic bases from their decomposition products, from inorganic salts, and from phenolic bacteriostats and preservatives.

Total Anesthetic.—Transfer a 25-ml. aliquot of the solution of extracted anesthetic bases to a 50-ml. volumetric flask, dilute to the mark with 7 *N* sulfuric acid, mix well. Determine the absorbance of the solution in a spectrophotometer at its absorption maximum about 272 mμ, using 25.0 ml. of 7 *N* sulfuric acid diluted to 50.0 ml. with water as a blank.

* Received August 9, 1958, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.
† Present address: Temple University, School of Pharmacy, Philadelphia 40, Pa.
[1] Trademark, Novocain (Winthrop Laboratories).
[2] Trademark, Pontocaine Hydrochloride (Winthrop Laboratories).
[3] Novocain-Pontocaine-Cobefrin (N. P. C.) Solution, available from the Cook-Waite Laboratories, 1450 Broadway, New York, N. Y.

% Total Anesthetic = $200\ A_{max.}/E^{1\%}_{1\ cm.}$ for procaine hydrochloride ($A_{max.}$ is the measured absorbance at the wavelength of maximum absorption).

With Beckman DU No. 61607, the absorption maximum for procaine hydrochloride is at 272 mμ with $E^{1\%}_{1\ cm.}$ = 35.6 in sulfuric acid ranging in concentration from 2 *N* to 7 *N*. In the same instrument, the absorption maximum for tetracaine hydrochloride is at 272 mμ with $E^{1\%}_{1\ cm.}$ = 34.5 in sulfuric acid ranging in concentration from 2 *N* to 7 *N*.

The absorbance of the sulfuric acid blank read against distilled water at 272 mμ is 0.000.

Obviously the recommended method for calculating total anesthetic introduces an error, since it ignores the fact that the absorbance of tetracaine hydrochloride is a little lower than that of procaine hydrochloride. However, for the commercial ratio of drugs, the error involved is not important. Calculation will show that for a solution actually containing 0.2150% total anesthetic, the experimental value (assuming no other errors), will be 0.2145%. The deliberate error therefore amounts to 5 parts in 2,150.

Tetracaine Hydrochloride.—Transfer a 50-ml. aliquot of the solution of extracted anesthetic bases to a separatory funnel, add 1 ml. of 7% sodium nitrite, mix well, add 1 ml. of 1 *N* hydrochloric acid, mix well, let stand for five minutes. Add 2 ml. of 10% sodium carbonate solution, mix, add 2 ml. of 1% Chicago acid, mix, let stand for one minute. Extract with three 25-ml. portions of chloroform, pooling the chloroform extracts in a separator. Wash the pooled chloroform extracts with 15 ml. of water. Filter the washed chloroform extracts through creped filter paper into a 100-ml. volumetric flask, dilute to the mark with chloroform, mix well. Determine the absorbance of the chloroform solution in a spectrophotometer at its absorption maximum (about 290 mμ) against a blank prepared by treating about 10 mg. of procaine hydrochloride in 50.0 ml. of water as described above.

% Tetracaine Hydrochloride = $200\ A_{max.}/E^{1\%}_{1\ cm.}$ for tetracaine hydrochloride after extraction and nitrosation.

One mg. of tetracaine hydrochloride, nitrosated, extracted with chloroform, and the extract diluted to 100.0 ml. had, in the Beckman DU No. 61607, an absorption maximum at 290 mμ. Six replicates had measured absorbances of 0.458, 0.455, 0.454, 0.456, 0.452, 0.455; average = 0.455; standard deviation by the method of Dean and Dixon (3) ± 0.002.

The absorbances of six blanks read against chloroform at 290 mμ were: 0.026, 0.028, 0.024, 0.024, 0.026, 0.026; average = 0.026; standard deviation ± 0.002.

Procaine Hydrochloride.—Subtract the per cent of tetracaine hydrochloride found from the per cent of total anesthetic found to obtain per cent of procaine hydrochloride.

If the solution is expected to contain a ratio of tetracaine to procaine hydrochloride of greater than 0.30 Gm. to 2.00 Gm., a more accurate calculation of procaine hydrochloride may be made as follows. Determine the absorbance at about 272 mμ as directed for Total Anesthetic, then use the following form for computation.

% Procaine Hydrochloride = $[200\ A_{max.} - (\%\ \text{Tetracaine HCl} \times E^{1\%}_{1\ cm.}\ \text{tetracaine HCl})]/E^{1\%}_{1\ cm.}$ procaine HCl

PRECISION AND ACCURACY

Six replicates of a solution made up to contain 20.000 mg. of procaine hydrochloride and 1.500 mg. of tetracaine hydrochloride per ml. were assayed with the results shown in Table I.

As an additional check of the method, aliquots of a commercial sample, deliberately partially decomposed by heating, were assayed by Mr. Pratt by his spectrophotometric nitrous acid titration, using a 20-ml. sample, whereas a 1-ml. sample was used in the method presented here. Results are given in Table II.

TABLE I.—MICROESTIMATION OF PROCAINE AND TETRACAINE HYDROCHLORIDES

Replicate No.	Total Anesthetic, % 2.15% Calculated	Tetracaine Hydrochloride, % 0.150% Calculated
1	2.16	0.149
2	2.13	0.149
3	2.15	0.151
4	2.13	0.148
5	2.14	0.151
6	2.14	0.150
Average	2.14	0.150
Standard deviation	±0.01	±0.001

TABLE II.—COMPARISON OF THE SPECTROPHOTOMETRIC TITRATION AND MICROMETHOD FOR THE ESTIMATION OF PROCAINE AND TETRACAINE HYDROCHLORIDES

	Procaine Hydrochloride Found, %	Tetracaine Hydrochloride Found, %
Spectrophotometric titration	2.06	0.125
Micromethod	2.03	0.129

DISCUSSION

The qualitative and quantitative changes in the ultraviolet absorption spectra of aromatic amines as pH of the solvent changed over the range of 2 to 12 have been noted by many authors. As early as 1950, however, the marked change in absorption spectrum of procaine as the acidity of the aqueous medium was increased from 0.01 *N* to 7 *N* was recognized in this laboratory.[4] This change in spectrum at high acid concentrations was found to be a property common to all *p*-aminobenzoic acid derivatives investigated. In neutral medium, these compounds have an absorption maximum at 300 ± 10 mμ, but in solutions of acidity greater than 1 *N* all except propoxycaine have an absorption maximum at 272 ±1 mμ. Propoxycaine, which differs from the other *p*-aminobenzoic acid anesthetics in having a propoxy group substituted on the benzene ring, has an absorption spectrum strikingly different from the other members of the group. It shows two absorption maxima

[4] To an important extent, the work of H. Wm. Eckert.

in neutral solution (at 280 and 303 mμ) and two in strongly acid solution (at 235 and 297 mμ).

In the assay of combinations of anesthetics derived from *p*-aminobenzoic acid and containing tetracaine, it should be noted that the chief chemical distinction between tetracaine and the anesthetics related to it is that tetracaine is a secondary aromatic amine, whereas the others are primary aromatic amines. This difference in structure is responsible for a difference in behavior toward nitrous acid. The primary amines react quickly and quantitatively to form diazonium salts; tetracaine reacts more slowly, but still quantitatively, to form the N-nitroso derivative.

The stability of N-nitroso compounds shows wide variation. N-Nitroso tetracaine is stable enough for use in the technique described, that is, in aqueous carbonate solution and in chloroform.

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Book Notices

The Health of a Nation. (Harvey W. Wiley and the Fight for Pure Food). By OSCAR E. ANDERSON, JR. The University of Chicago Press, Chicago, 1958. ix + 333 pp. 15.5 x 23.5 cm. Price $6.

This book covers the history of the long fight for progressive food legislation which brought about the Federal Food and Drugs Law of 1906, and of the continuing struggle which resulted in attempting to administer its provisions. The main emphasis, quite properly, is on the life and accomplishments of Dr. Harvey W. Wiley, who, as chief of the Department of Agriculture's Bureau of Chemistry, marshaled the forces for federal regulation of the food and drug industries, and later served as chief administrative officer of the 1906 Food and Drugs Act.

Proceedings of the National Conference on Hospital-Acquired Staphylococcal Disease. Sponsored by U. S. Public Health Service—Communicable Disease Center—National Academy of Sciences—National Research Council. U. S. Dept. of Health, Educ., and Welfare, Atlanta, 1958. xi + 178 + xxv pp. 20.5 x 26.5 cm.

Papers and discussions presented during a national conference on hospital-acquired staphylococcal disease.

Poliomyelitis. Compiled and Edited for the International Poliomyelitis Congress. J. B. Lippincott Co., Philadelphia, 1958. xviii + 684 pp. 17.5 x 25.5 cm. Price $7.50.

Papers and discussions presented at the Fourth International Poliomyelitis Conference.

Catalysis. Vol. VI. Edited by PAUL H. EMMETT. Reinhold Publishing Corp., New York, 1958. vi + 706 pp. 15 x 23 cm. Price $19.50.

This sixth volume covers catalytic cracking, isomerization of hydrocarbons, polymerization, polymerization of olefins, alkylation, and hydro-reforming.

This book, together with the other volumes of the series, should prove of tremendous value to physical chemists and to every industry in which catalysis is involved.

Volkstümliche Namen der Arzneimittel, Drogen, Heilkräuter und Chemikalien. Vierzehnte Auflage. Edited by JOHANNES ARENDS. Springer-Verlag, Berlin, Göttingen, Heidelberg, 1958. v + 411 pp 13 x 18.5 cm. Price DM 15.

A dictionary of German vernacular drug names for older botanicals and medicinal chemicals.

Biological Laboratory Data. By L. J. HALE. John Wiley & Sons, Inc., New York, 1958. x + 132 pp. 12 x 18.5 cm. Price $2.75.

A handbook of mathematical, physical, chemical, statistical, and other data used frequently by biologists.

Essentials of Therapeutic Nutrition. By SOLOMON GARB. Springer Publishing Co., Inc., New York, 1958. ix + 147 pp. 14 x 21 cm. Price $2.

An outline of the principles of nutrition, therapeutic diets, and reference tables designed especially for nurses.

A History of the American Soft Drink Industry. By JOHN J. RILEY. American Bottlers of Carbonated Beverages, Washington, D. C., 1958. xii + 302 pp. 15 x 22.5 cm. Price $10.

Outlines early efforts in Europe during the 1700's to duplicate the effervescent waters of the natural springs, and traces initial European developments. The interest in the United States in the same subject since 1800 is also described, followed by the origin of its commercial aspects when the artificially carbonated waters were sold primarily in drugstores. The part played by pharmacists in the early development of the soft drink industry in this country is emphasized throughout.

Social Aspects of Psychiatry. Edited by BENJAMIN PASAMANICK and PETER H. KNAPP. Psychiatric Research Reports, American Psychiatric Assoc., Washington 9, D. C., 1958. 208 pp. 15 x 23 cm. Paperbound. Price $2.

Consists of a series of papers presented to the Regional Research Conference held at Columbus, Ohio, on February 24–25, 1958.

CORRECTION

Change the name of the author of "Phosphorus and Its Compounds" in the Book Notices section [THIS JOURNAL, **48,** 133(1959)] to read: John R. Van Wazer instead of John R. Van Waxer.

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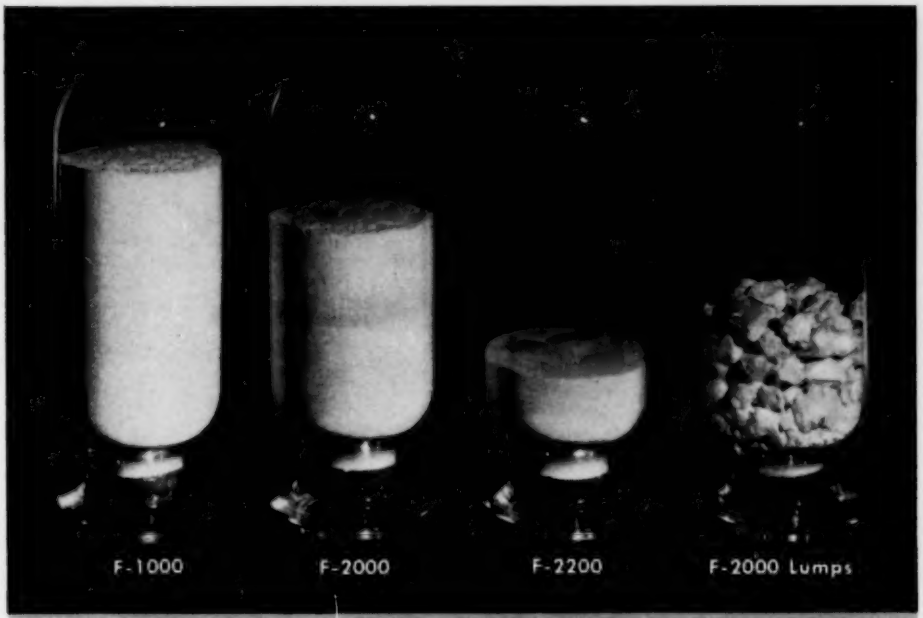

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